# Induction of Pre-Infection Thread Structures in the Leguminous Host Plant by Mitogenic Lipo-Oligosaccharides of *Rhizobium*

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**Root nodules of leguminous plants are symbiotic organs in which *Rhizobium* bacteria fix nitrogen. Their formation requires the induction of a nodule meristem and the formation of a tubular structure, the infection thread, through which the rhizobia reach the nodule primordium. In the *Rhizobium* host plants pea and vetch, pre-infection thread structures always preceded the formation of infection threads. These structures consisted of cytoplasmic bridges traversing the central vacuole of outer cortical root cells, aligned in radial rows. In vetch, the site of the infection thread was determined by the plant rather than by the invading rhizobia. Like nodule primordia, pre-infection thread structures could be induced in the absence of rhizobia provided that mitogenic lipo-oligosaccharides produced by *Rhizobium leguminosarum* biovar viciae were added to the plant. In this case, cells in the two outer cortical cell layers containing cytoplasmic bridges may have formed root hairs. A common morphogenetic pathway may be shared in the formation of root hairs and infection threads.**

*Rhizobium leguminosarum* biovar viciae bacteria form root nodules in symbiotic association with plants such as pea (*Pisum sativum*) and vetch (*Vicia sativa* ssp. *nigra*). In these plants, formation of a nodule primordium is induced in the inner cortex of the host root at some distance from the advancing infection thread (*1–4*). In alfalfa (*5, 6*) and vetch (*7*), mitogenic lipo-oligosaccharide molecules, produced by infective homologous rhizobia, are responsible for the induction of cell divisions leading to the formation of a root nodule primordium. After root hair infection, *Rhizobium* bacteria migrate intracellularly toward the dividing root cells via a tubular structure, the infection thread, which elongates by growth at the tip (*8*). Induction and maintenance of a tip-growth process in a plant cell—for instance during formation of a root hair—requires a polarized cytoplasmic organization (*9*). It has not been known to what extent root infection or the presence of rhizobia in a growing infection thread influences cell polarization and thereby infection thread growth in a legume root. We therefore compared the behavior of root cortical cells after inoculation with infective bacteria and after addition of lipo-oligosaccharide signal molecules by using light and transmission electron microscopy.

At an early stage of root nodule development in vetch and pea (*10, 11*), all root cortical cells in the sector between a root hair infected by rhizobia and the central cylinder of the root respond almost simultaneously with morphological changes. Cell nuclei swell and migrate to the center of the cell. In pea, these changes are accompanied by expression of the host gene product ENOD12 (*4*). In vacuolated plant cells, swelling of the nucleus and its migration to the cell center usually precede cell division (*12*). However, in vetch and pea (*10, 11*), cell divisions occur only in cells located in the inner root cortex. Usually, initial cell divisions appear to be radial, resulting in radial rows of cells with radially positioned division walls (Fig. 1A). Further cell divisions result in the initiation of a nodule primordium. In the outer cortical cells of the infection zone, swelling of the cell nucleus and its migration to the center of the cell are not followed by cell division. Instead, the cytoplasm collects to form a radially oriented conical structure, which we designate as a cytoplasmic bridge (*10, 11*) (Figs. 1 and 2). Cytoplasmic bridges in cells of the outer cortex are usually positioned in line with young radial division walls in the inner cortex, which results in the formation of very typical radial rows of cells characterized by the presence of these aligned radially oriented structures (Fig. 1A). The infection thread appears to grow through and within cytoplasmic bridges (Fig. 1C). Therefore, we propose to call these aligned bridges pre-infection thread structures (Pit). The cytoplasmic bridges are polarized: the bulk of cytoplasm and endomembranes are located at the outer side, and all amyloplasts are at the inner side of the bridge.

Nodule primordia are induced by two mitogenic lipo-oligosaccharides produced by *Rhizobium leguminosarum* bv. viciae, NodRlv-IV(Ac,C18:4) and NodRlv-V (Ac,C18:4) (*7, 13*). Both molecules are characterized by the presence of a highly unsaturated lipid moiety and an O-acetyl group. Each of these two nodulation (Nod) factors can induce root nodule primordia in *Vicia sativa* ssp. *nigra* roots (*7*). Nonmitogenic Nod factors that differed in the acyl substituent group were designated NodRlv-IV(Ac,C18:1) and NodRlv-V(Ac,C18:1), whereas those that lacked an O-acetyl group were designated NodRlv-IV(C18:4) and NodRlv-V(C18:4) (*7*). These Nod factors do not induce nodule primordia in *V. sativa* ssp. *nigra* roots.

Roots of axenically cultured *V. sativa* ssp. *nigra* plants were tested for response to rhizobial lipo-oligosaccharides. *Vicia sativa* ssp. *nigra* is more suitable than pea for bioassays related to root nodule symbiosis because of its smaller size and its larger number of nodulation-related phenotypes (*2, 3, 7, 14, 15*). Addition of a crude Nod factor extract [containing NodRlv-IV(Ac, C18:4), NodRlv-V(Ac,C18:4), NodRlv-IV (Ac,C18:1), and NodRlv-V(Ac,C18:1)] from *R. leguminosarum* strain RBL5601 that has a complete set of *nod* genes or either of the purified mitogenic Nod factors NodRlv-IV(Ac,C18:4) or NodRlv-V(Ac,C18:4) resulted in a response of root cortical cells identical to that found after inoculation with infective bacteria and in the same zone of the root. Inner cortical cells showed radial cell divisions and outer cortical cells formed cytoplasmic bridges. In addition, radial rows of responding cells similar to those seen in *Rhizobium*-infected roots were formed (Fig. 1, A and B). These responses were absent if the plants were treated under identical conditions with the nonmitogenic Nod factors.

The formation of mitogenic Nod factor–induced, cytoplasmic bridges in cells of the two outermost cortical cell layers of the root is normally accompanied by local weakening of the outer periclinal wall and frequently by root hair formation by these cortical cells (Fig. 1D). In front of 12 out of the 24 Nod factor–induced root nodule primordia we investigate, root hairs formed by outer cortical cells were observed. Normally, root hairs are formed only by plant root epidermal cells as tubular protuberances elongating by growth at the tip (*16*). Like infection thread formation, the initiation of root hair growth requires local modifications of the outer periclinal cell wall and is assumed to be preceded by intracellular polarization (*9*). As in root hair formation, a cytoplasmic bridge may be involved in the initiation of such cell wall modifications for infection (Fig. 1D). A possibly weakened, deformed area could be observed in the cell wall separating the

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approaching infection thread from the cytoplasmic bridge (Fig. 1C). Such cell wall modifications were normally seen in front of nodule meristems in *Rhizobium*-infected *Vicia* roots, investigated by light microscopy. These observations, and the fact that *Rhizobium* initiates infection threads in its host plant at young, emerging root hair tips (*11*), are consistent with the hypothesis that root hair formation and infection thread formation follow a common morphogenetic pathway.

Induction of root hair formation by sterilized supernatant fluids of *R. leguminosarum* bv. viciae cultures has been reported (*17*). This kind of root hair formation takes place over the entire surface of the elongating part of the root and not only in front of the root nodule primordia (Hai, for root hair induction). In *V. sativa* ssp. *nigra*, Hai is also induced by *Rhizobium* mutants that do not produce mitogenic factors (*17*) and by physical and chemical stress factors (*18*). Thin sectioning revealed that these root hairs were of epidermal origin (*18*). Apparently, nonmitogenic Nod factors are sufficient for enhancement of root hair formation by the preconditioned cells, whereas mitogenic Nod factors are necessary for induction of root hair formation in cortical cells. Induction of cytoplasmic bridge formation in vetch by Nod factors produced by *nodH*$^-$ mutants of *Rhizobium meliloti* (*15*) has yet to be tested.

Our results show that in vetch, the plant, and not the invading rhizobia, determines the site where the infection thread is formed. The ability of a Nod factor to induce cell polarization and Pit is correlated with its ability to induce cell divisions (Fig. 2). Libbenga and co-workers (*19*) proposed that the gradient hypothesis for root nodule initiation in pea—that is, induction of cell divisions in the inner root cortex—is determined by endogeneous gradients of both plant growth factors and rhizobial stimuli. We propose (Fig. 2) that cell polarization in the outer cortex is also determined by these gradients. Such gradients are different in plants like soybean and bean because early root nodule initiation in these plants takes place in the outer cortex instead of in the inner cortex (*11*). Furthermore, another type of infection thread is formed (*11*), and Pit may not occur during nodulation of these legumes.

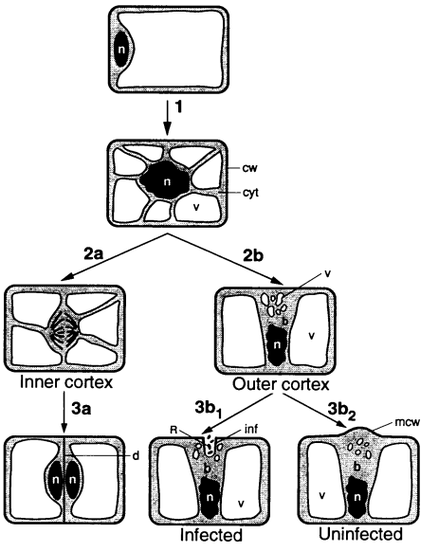


**Fig. 2.** Model depicting the effects of mitogenic Nod factors on the activated cortical cells of a vetch root. Stage 1: In all cells, the nuclei enlarge and move to the center of the cell, being connected to the parietal cytoplasm by cytoplasmic strands. Stage 2: (a) In the inner cortex, the cells continue the cell division cycle and divide; in (b), the cells in the outer cortex do not divide but instead form cytoplasmic bridges. Stage 3: (a) Inner cortical cells have divided and can enter the cell cycle again. (b) In the outer cortical cells, the cell wall is modified at the outer side of the bridge, resulting in the formation of an infection thread if *Rhizobium* is present ($3b_1$) or in the formation of a root hair if only a mitogenic Nod factor is present ($3b_2$). Symbols: cw, cell wall; n, nucleus; cyt, cytoplasm; v, vacuole; b, cytoplasmic bridge; d, division wall; R, *Rhizobium* bacteria; inf, infection thread; mcw, modified cell wall.

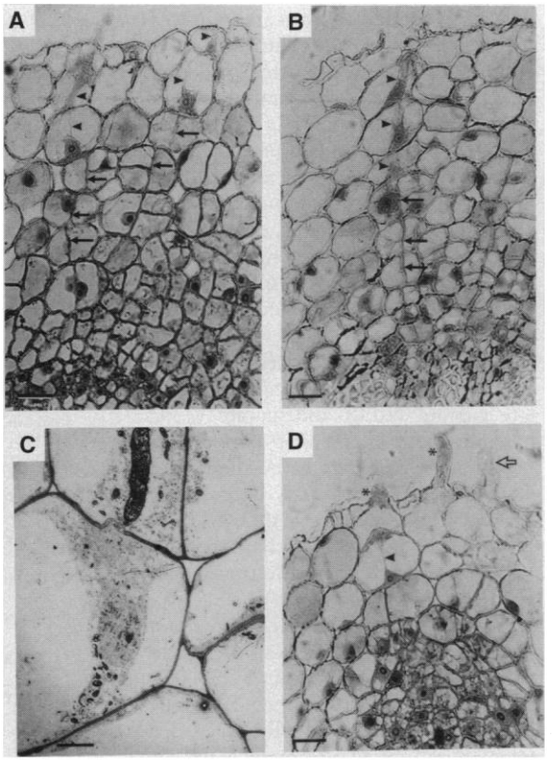


**Fig. 1.** Pre-infection thread structures (*20*). (**A** and **B**) Transverse sections of 4-day-old vetch roots, each showing a young root nodule primordium and pre-infection thread structures. In (A), induction by *R. leguminosarum* bv. viciae; in (B), induction by NodRlv-V(Ac,C18:4) factor. Bars represent 20 μm. Each section shows radial rows of responding cells with anticlinally positioned cell walls (arrows) in the inner cortical cells, in line with similarly positioned cytoplasmic bridges of the pre-infection thread structure (arrowheads) in the outer cortical cells. Similar results were obtained after addition of NodRlv-IV(Ac,C18:4) factor or crude wild-type Nod factors from strain RBL5601. (**C**) Electron micrograph of outer cortical cells in a transversely sectioned pea root (*20*) after inoculation with *R. leguminosarum* bv. viciae. An advancing infection thread that contained bacteria is present in the upper cell, and a cytoplasmic bridge can be seen in the subadjacent cell. In the center of the outer periclinal cell wall and at the axis of the cytoplasmic bridge, the presumptive future crossing site of the infection thread can be recognized as a less electron-dense, wrinkled region, which may be weaker than the surrounding cell wall. Bar represents 10 μm. (**D**) Transverse section of a 5-day-old vetch root showing a root nodule primordium and root hair formation induced by the NodRlv-V(Ac,C18:4) factor (*20*). An apparent plasticity of the outer periclinal wall of one cell can be observed at the contact area with the cytoplasmic bridge (arrowhead). Two root hairs originating from outer cortical cells are present (asterisks). A genuine root hair, originating from an epidermal cell, is out of focus (open arrow). Bar represents 20 μm.

## REFERENCES AND NOTES


1. T. Oinuma, *Seitbusu* **3**, 155 (1948).
2. D. A. Phillips, *Physiol. Plant.* **25**, 482 (1971).
3. K. R. Libbenga and P. A. A. Harkes, *Planta* **114**, 17 (1973).
4. B. Scheres *et al.*, *Cell* **60**, 281 (1990).
5. P. Lerouge *et al.*, *Nature* **344**, 781 (1990).
6. G. Truchet *et al.*, *ibid.* **351**, 670 (1991).
7. H. P. Spaink *et al.*, *ibid.* **354**, 125 (1991).
8. P. J. Dart, in *The Biology of Nitrogen Fixation*, A. Quispel, Ed. (North-Holland, Amsterdam, 1974), pp. 381–429.
9. A. Sievers and E. Schnepf, in *Cytomorphogenesis in Plants*, O. Kiermayer, Ed. (Springer-Verlag, Berlin, 1981), pp. 263–299.
10. R. Bakhuizen, thesis, Leiden University, Leiden, The Netherlands (1988).
11. J. W. Kijne, in *Biological Nitrogen Fixation*, G. Stacey, R. H. Burris, H. J. Evans, Eds. (Chapman and Hall, New York, 1992), pp. 349–398.
12. R. Bakhuizen, P. C. van Spronsen, F. A. J. Sluiman-den Hartog, C. J. Venverloo, L. Goosen-de Roo, *Protoplasma* **128**, 43 (1985).
13. The two mitogenic lipo-oligosaccharides produced by *R. leguminosarum* bv. viciae are *N,N′,N″*-triacetyl-*N‴*-(*trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoyl)-6‴-*O*-acetyl-chitotetraose [abbreviated NodRlv-IV(Ac,C18:4)] and *N,N′,N″,N‴*-tetraacetyl-*N⁗*-(*trans*-2,*trans*-4,*trans*-6,*cis*-11-

octadecatetraenoyl)-6""-*O*-acetyl-chitopentaose [abbreviated NodRlv-V(Ac,C18:4)] (*7*). Both molecules are characterized by the presence of a highly unsaturated lipid moiety and an *O*-acetyl substituent. Besides these two nodulation signals, other Nod metabolites were reported (*7*) that differ in the acyl substituent or that lack the *O*-acetyl group. When the acyl substituent is *cis*-vaccenic acid, the corresponding Nod factors are designated NodRlv-IV(Ac,C18:1) and NodRlv-V(Ac,C18:1). When the *O*-acetyl substituent is absent, the corresponding Nod factors are designated NodRlv-IV(C18:4) and NodRlv-V(C18:4). A Tn*5* mutation in the rhizobial nodulation gene *nodE* causes the absence of the NodRlv-IV(Ac,C18:4) and NodRlv-V(Ac,C18:4) Nod factors, and instead the corresponding Nod factors NodRlv-IV(Ac,C18:1) and NodRlv-V(Ac,C18:1) are formed (*7*). A *nodL*::Tn*5* mutation results in the absence of the *O*-acetyl group in all Nod factors produced, such as NodRlv-IV(C18:4) and NodRlv-V(C18:4) (*7*).

14. H. C. J. Canter Cremers, A. A. N. van Brussel, J. Plazinski, B. G. Rolfe, *J. Plant Physiol.* **122**, 25 (1986).
15. P. Roche *et al.*, *Cell* **67**, 1131 (1991).
16. R. G. H. Cormarck, *Bot. Rev.* **15**, 446 (1962).
17. S. A. J. Zaat, A. A. N. van Brussel, T. Tak, E. Pees, B. J. J. Lugtenberg, *J. Bacteriol.* **169**, 3388 (1987).
18. A. A. N. van Brussel, R. Bakhuizen, P. C. van Spronsen, H. P. Spaink, T. Tak, B. J. J. Lugtenberg, J. W. Kijne, unpublished results.
19. K. R. Libbenga, F. van Iren, R. J. Bogers, M. F. Schraag-Lamers, *Planta* **114**, 29 (1973).
20. Methods used for Fig. 1, A, B, and D: Induction of nodulation phenotypes by *R. leguminosarum* bv. viciae bacteria, crude Nod factors, or purified Nod factors was tested with *Vicia sativa* ssp. *nigra* plants in glass containers with the roots shielded from light (*7*). *Rhizobium leguminosarum* bv. viciae strain RBL5601 (Nod$^+$Fix$^+$) was grown for 3 days on solidified yeast mannitol broth medium [P. J. J. Hooykaas, P. M. Klapwijk, M. P. Nuti, R. A. Schilperoort, A. Rörsch, *J. Gen. Microbiol.* **98**, 477 (1979)], suspended in sterile water, and added to the *Vicia* plant growth medium at $10^5$ to $10^6$ cells per milliliter. Crude Nod factors were extracted with the use of butanol (*7*) from *Rhizobium* cultures of the strains RBL5601 (containing the Sym plasmid pRL1JI) for wild-type crude Nod factors, RBL5602 (pRILJI*nodE*::Tn*5*) for crude Ac,C18:1 Nod factors, and RBL5793 (pRL1JI*nodL*589::Tn*5phoA*) for crude C18:4 Nod factors [A. A. N. van Brussel *et al.*, *J. Bacteriol.* **172**, 5394 (1990)]. The butanol phase was evaporated, and the residue was redissolved in an acetonitrile and water mixture (1:1, v/v; 25 ml per liter of the original culture medium). Nod factors produced by *R. leguminosarum* bv. viciae strain RBL5601 were purified as described (*7*). Purified and crude Nod factors were tested as follows. Samples (100 μl) of solutions in acetonitrile-water (1:1, v/v) were evaporated under sterile conditions at 80°C. Sterile medium (20 ml) was added, yielding final factor concentrations of $10^{-7}$ M for the purified Nod factor and a final concentration of the crude Nod factor corresponding to one-fifth of the concentration in the *Rhizobium* culture from which the factors were extracted. Crude Nod factor samples containing the lipooligosaccharides NodRlv-IV(Ac,C18:1), NodRlv-V(Ac,C18:1) or NodRlv-IV(C18:4), and NodRlv-V(C18:4) required solubilization in sterile 1% CHAPS (Sigma) (*7*). Seedlings were mounted in glass containers, and after 4 or 5 days of incubation with *Rhizobium* or with Nod factors, the roots were processed for thin sectioning as described (*12*), with the exception that the first and second fixation periods were reduced to 1 hour and 45 min, respectively. Transverse serial sections of 8 μm thickness were made of the developing nodule meristems and investigated by light microscopy. For Fig. 1C, induction of nodulation phenotypes was tested with pea seedlings grown and inoculated with *Rhizobium leguminosarum* bv. viciae strain 248 (Nod$^+$Fix$^+$) as described [C. L. Diaz *et al.*, *Planta* **168**, 350 (1986)]. The roots were processed for electron microscopy (*12*), and serial thin sections were examined with a Philips 300 electron microscope operating at 60 kV.
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# Fatty Acid Biosynthesis Redirected to Medium Chains in Transgenic Oilseed Plants

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**Medium-chain fatty acids (FAs), found in storage lipids of certain plants, are an important renewable resource. Seeds of undomesticated California bay accumulate laurate (12:0), and a 12:0–acyl-carrier protein thioesterase (BTE) has been purified from this tissue. Sequencing of BTE enabled the cloning of a complementary DNA coding for a plastid-targeted preprotein. Expression of the complementary DNA in the seeds of *Arabidopsis thaliana* resulted in BTE activity, and medium chains accumulated at the expense of long-chain (≥16) FAs. Laurate became the most abundant FA species and was deposited in the storage triacylglycerols. These results demonstrate a mechanism for medium-chain FA synthesis in plants.**

In plants, FAs are assembled in the plastids where the fatty acid synthase (FAS) sequentially condenses two-carbon units onto the growing fatty acyl chain. Whereas the end products are usually 16- or 18-carbon FAs (*1*), members of several plant families synthesize large amounts of predominantly 8- to 14-carbon (medium-chain) FAs. Some are harvested for dietary or industrial purposes—for example laurate, which is currently extracted from the seeds of tropical trees at a rate approaching one million tons annually (*2*). We investigated the feasibility of producing laurate in an annual, temperate crop by genetic engineering.

In the developing oilseeds of California bay (*Umbellularia californica*) that accumulate caprate (10:0) and laurate (12:0), a medium-chain acyl acyl-carrier protein (acyl-ACP) BTE has been identified (*3*). This BTE, by prematurely hydrolyzing the growing acyl thioesters, is thought to play a critical role in medium-chain production (*3*). We report here on the transfer of this activity into seeds of *A. thaliana*.

Peptide sequences of BTE were obtained (*4*, *5*), enabling the synthesis of primers for polymerase chain reaction (PCR) amplification of reverse-transcribed BTE mRNA (*6*). The PCR-derived BTE DNA sequence fragment was used to obtain a full-length cDNA (*7*). The ATG nearest the 5′ end of the single, large open reading frame is surrounded by a sequence matching the motif for initiation of translation in plants (*8*). A 382–amino acid polypeptide (molecular weight 33782) containing most of the BTE peptide sequences (*5*) was predicted (Fig. 1).

Mature BTE, when isolated from developing seeds, appears to be a processed form of the predicted polypeptide, having its $NH_2$-terminus at amino acid 84 (Fig. 1) (*5*). A search of all currently available gene banks with the derived amino acid sequence yielded no significant matches. Using ribonuclease protection, we detected no BTE transcripts in bay leaves. In developing cot-

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MATTSLASAFCSMKAVMLARDGRGMKPRSSDLQLRAGNAP  40
TSLKMINGTKFSYTESLKRLPDWSMLFAVITTIFSAAEKQ  80
WTNLEWKPKPKLPQLLDDHFGLHGLVFRRTFAIRSYEVGP 120
DRSTSILAVMNHMQEATLNHAKSVGILGDGFGTTLEMSKR 160
DLMWVVRRTHVAVERYPTWGDTVEVECWIGASGNNGMRRD 200
FLVRDCKTGEILTRCTSLSVLMNTRTRRLSTIPDEVRGEI 240
GPAFIDNVAVKDDEIKKLQKLNDSTADYIQGGLTPRWNDL 280
DVNQHVNNLKYVAWVFETVPDSIFESHHISSFTLEYRREC 320
TRDSVLRSLTTVSGGSSEAGLVCDHLLQLEGGSEVLRART 360
EWRPKLTDSFRGISVIPAEPRV 382
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**Fig. 1.** Amino acid sequence of bay 12:0-ACP TE derived from the cDNA of pCGN3822 (*7*). The $NH_2$-terminus of the BTE, as obtained from the purified protein (*5*), is indicated by a dot below the respective amino acid. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. GenBank accession number M94159.

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