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The PsENOD12 Gene Is Expressed at Two Different Sites in Afghanistan Pea Pseudonodules Induced by Auxin Transport Inhibitors[1]

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ABSTRACT

A number of early nodulin genes are expressed in specific cell types as pea (*Pisum sativum*) root nodules develop. The *Pisum sativum* early nodulin PsENOD2 is detected only in the uninfected cells of the nodule parenchyma, whereas PsENOD12 is expressed at two spatially removed sites: in root hairs and adjacent cortical cells, both of which can be invaded by *Rhizobium* entering through infection threads, and in derivatives of newly divided root inner cortical cells that establish the nodule primordium. We tested whether *Rhizobium* infection is required for triggering PsENOD12 gene expression by inducing nodule-like structures on Afghanistan pea roots with the auxin transport inhibitor *N*-(1-naphthyl)phthalamic acid (NPA). These nodule-like structures lack infection threads but resemble *Rhizobium*-induced nodules in other aspects. For one, both PsENOD2 and PsENOD12 transcripts were detected in these structures. PsENOD2 mRNA was localized by in situ hybridization to a zone equivalent to the nodule parenchyma of *Rhizobium*-induced nodules, whereas PsENOD12 transcripts were detected in a group of cells comparable to the nodule primordium of developing nodules. In addition, PsENOD12 mRNA was detected in uninfected root hairs 48 h after NPA treatment. These results indicate that infection is not a trigger for PsENOD12 gene expression in Afghanistan pea and rather suggest that the expression of the PsENOD2 and PsENOD12 genes is correlated with the differentiation of specific cell types in the developing nodule.

Rhizobium nodulation (*nod*) genes are essential for root hair curling, bacterial infection, and initiation of a nodule primordium (for review, see ref. 10). Upon induction of the *nod* genes, the bacteria secrete molecules called Nod factors, which elicit root hair deformation (8). Lerouge et al. (19) characterized the Nod factor of *R. meliloti* as a sulfated β-linked tetraglucosamine with acetyl and acyl substitutions. Substituted penta- and triglucosamine Nod factors have also been identified from *R. meliloti* (26). *R. leguminosarum* bv. *viciae*, the bacterium that nodulates pea and vetch, produces Nod factors that are structurally related to those of *R. meliloti* (27). Nod factors from both *R. meliloti* and *R. leguminosarum* bv. *viciae* elicit root hair deformation and cortical cell divisions in alfalfa and vetch, respectively (19, 27). In addition, *R. meliloti* Nod factor induces small, bacteria-free nodules on alfalfa roots (29).

Previously, we reported that compounds that function as ATIs[5], e.g. TIBA and NPA, induced nodule-like structures on alfalfa roots (14). The ATIs, which are modified benzoic acids and structurally unrelated to the substituted lipo-oligosaccharides made upon induction of the *R. meliloti nod* genes, trigger the development of nodule-like structures in a number of legumes (1). These pseudonodules share a number of histological features with *Rhizobium*-induced root nodules, including the presence of a tissue equivalent to nodule parenchyma, a central tissue, and a nodule cortex (1, 14). They also exhibit several major structural differences, e.g. the lack of a discrete, distally positioned nodule meristem and the absence of peripheral vascular bundles (14, 30). Like *Rhizobium*-induced nodules, pseudonodules induced by TIBA and NPA contain transcripts for two nodulin genes: MsENOD2 (7), an alfalfa early nodulin gene, and Nms30 (7, 24), known only as an in vitro translation product (14). Early nodulins are proteins expressed during root hair deformation, infection, and nodule morphogenesis, whereas the late nodulins are proteins involved in nodule function or maintenance, such as leghemoglobin and glutamine synthetase (13). Thus, the ATIs mimic *R. meliloti* Nod factors in that they induce cell divisions, pseudonodule morphogenesis, and the expression of several early nodulin genes in alfalfa.

In the pea *R. leguminosarum* bv. *viciae* symbiosis, PsENOD2 and PsENOD12 have been used as molecular markers to track the early stages of nodule development. PsENOD2 transcripts are first detected in nodule parenchyma cells of pea nodule primordia 5 d after inoculation with *R. leguminosarum* bv. *viciae* (31). PsENOD12 is expressed in root hairs 48 h after inoculation with rhizobia or after incubation with

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[5] Abbreviations: ATI, auxin transport inhibitor; NPA, *N*-(1-naphthyl)phthalamic acid; TIBA, 2,3,5-triiodobenzoic acid; PsENOD, *Pisum sativum* Early Nodulin.

a *R. leguminosarum* bv. *viciae* culture supernatant containing Nod factors (25). Induction of PsENOD12 gene expression in root hairs by rhizobia or by medium in which rhizobia have grown depends upon the presence of the common *nod* genes (25).

Forty-eight hours after inoculation of pea roots with *R. leguminosarum* bv. *viciae*, PsENOD12 is expressed in two physically separated areas: (a) in root hair and adjacent cortical cells invaded by or in the path of infection threads; and (b) in newly divided root inner cortical cells that establish the nodule primordium (25). This disparate localization soon after inoculation suggests that PsENOD12 has a function not only in the infection process, but also in nodule primordium formation. Here, we address the question as to whether PsENOD12 is expressed in the absence of an infection process by treating pea roots with ATIs. Because the ATIs mimic Nod factor by inducing cell divisions and early nodulin gene expression, we first investigated whether the ATIs, although structurally unrelated to the substituted lipo-oligosaccharides that cause root hair deformation, could elicit PsENOD12 gene expression in pea root hairs. We then asked whether the ATIs mimic the *nod* gene products by inducing PsENOD12 expression at a second site, the nodule primordium.

MATERIALS AND METHODS

Plant Materials

NPA Incubation

Pisum sativum L. cv Rondo was grown as described by Bisseling et al. (2). Sterilized seeds of *P. sativum* cv Afghanistan were planted in autoclaved plastic dish pans containing perlite covered by 1 cm of vermiculite watered with Jensen's medium (32) containing 20 to 25 mM KNO_3. The Afghanistan pea seeds were surface-sterilized in 95% ethanol for 1 h and 5.25% sodium hypochlorite for 30 min, followed by copious rinsing with sterile distilled water. The seedlings that resulted were not damaged by these sterilization conditions. Two weeks after sowing, a solution consisting of 10^{-5} M NPA in 1 L of Jensen's medium containing 25 mM KNO_3 was added to the seedlings. Nodule-like structures were harvested 2 to 4 weeks later. Some nodules were surface sterilized and checked for the presence of bacteria (18).

Bacterial Incubation

Sterilized Rondo seeds were inoculated with *R. leguminosarum* bv. *viciae* 248, and nodules were harvested 14 to 17 d later. *P. sativum* cv Afghanistan was inoculated with *R. leguminosarum* bv. *viciae* TOM. Nodules were collected 2 to 3 weeks after inoculation.

Root Hair Analysis

Three-day-old Rondo and Afghanistan seedlings were inoculated either with *R. leguminosarum* bv. *viciae* 248 or TOM, respectively, or treated with 10^{-5} M NPA. One hundred fifty plants were used per inoculation or NPA treatment. The seedlings were harvested 2 d postinoculation or NPA treatment. Root hairs were isolated as described by Gloudemans et al. (12).

Detection of PsENOD12 Transcript

Total RNA from *Rhizobium*-induced nodules, NPA-induced pseudonodules, and root hairs was isolated by phenol extraction and LiCl precipitation according to DeVries et al. (6). RNA concentration was measured spectrophotometrically. For RNA transfer blotting, 10 μg of total RNA were subjected to electrophoresis and blotted onto GeneScreen (New England Nuclear) membranes as described by Franssen et al. (11), or in some experiments, 1 μg of poly(A)$^+$ RNA was fractionated in 1% agarose containing 6.7% formaldehyde (22) and transferred to Nytran (Schleicher and Schuell) membranes. Blots were hybridized to [α-^{32}P]dATP-labeled PsENOD2 (31) and PsENOD12 (25) cDNA inserts using random primers as described by Feinberg and Vogelstein (9). For detection of PsENOD12 in root hairs, the polymerase chain reaction was employed as described by Scheres et al. (25). A synthetic primer complementary to one strand of the 3′ end of the PsENOD12 cDNA sequence was used to synthesize PsENOD12 cDNA in total root hair RNA preparations. This cDNA was amplified after the addition of a second primer complementary to the 5′ end with *Taq* polymerase (Cetus) in 12 cycles of denaturation, annealing, and extension. The amplified cDNA was detected after Southern blotting and hybridization with ^{32}P-labeled PsENOD12 insert as described (25).

In Situ Hybridization

^{35}S-labeled antisense RNA probes transcribed from the plasmids pPsENOD2 and pPsENOD12 were synthesized as described (25, 31). In situ hybridization on Paraplast-embedded nodules was performed as described (4, 30). Sections were stained with toluidine blue. PsENOD12 transcripts were also localized with the RNA detection "Genius" Kit (Boehringer-Mannheim) on 8-μm thick paraffin sections of NPA-induced pseudonodules and nitrogen-fixing nodules (3). The sections were not stained. More than 40 different nitrogen-fixing nodules and pseudonodules were examined with each RNA probe.

RESULTS

Effect of NPA on Pea Roots

NPA induces pseudonodules on alfalfa (*Medicago sativa*) (14), *M. truncatula*, and species of *Melilotus* (A.M. Hirsch, unpublished results), as well as clover (*Trifolium repens*) (28). Allen et al. (1) found that a number of legumes, including cowpea and soybean, responded to ATI treatment by forming root swellings or pseudonodules. We tested whether five different pea cvs, Rondo, Little Marvel, Alaska, Sparkle, and Afghanistan, would form pseudonodules after NPA treatment. NPA induced nodule-like structures on fewer than 3% of cv Alaska plants at 10^{-5} M, the most effective concentration for inducing pseudonodules on alfalfa and clover. At the same concentration, the cvs Sparkle and Little Marvel developed terminal root swellings but no nodule-like structures. Only cv Afghanistan consistently formed pseudonodules in response to 10^{-5} M NPA. Approximately 60% of the plants developed pseudonodules on the primary root. This differs

from *Rhizobium*-induced nodules, which normally develop on the secondary roots. When cv Afghanistan roots were treated with 10^{-6} M NPA, fewer than 5% of the plants formed pseudonodules.

PsENOD12 Gene Expression in Root Hairs upon NPA Treatment

To determine whether NPA could induce PsENOD12 gene expression in root hairs, 3-d-old seedlings of *P. sativum* cvs Rondo and Afghanistan were treated with 10^{-5} M NPA. Forty-eight hours after treatment, the root hairs were harvested. When treated plants were examined under the microscope, none of the root hairs was found to be deformed. RNA of root hairs from NPA-treated plants as well as from untreated plants and roots inoculated with *R. leguminosarum* were analyzed for the presence of PsENOD12 mRNA by the polymerase chain reaction.

PsENOD12 transcripts could be detected in root hairs of cvs Rondo and Afghanistan 48 h after inoculation with *R. leguminosarum* bv. *viciae* strains 248 and TOM, respectively, whereas no PsENOD12 mRNA was detectable in root hairs from uninoculated plants (Fig. 1). When seedlings of cvs Rondo and Afghanistan were treated with 10^{-5} M NPA, we detected PsENOD12 transcripts in root hair RNA 48 h after treatment (Fig. 1), although at lower levels than when roots were inoculated with *R. leguminosarum* bv. *viciae* for the same duration of time. We concluded that NPA can induce PsENOD12 gene expression in pea root hairs.

Nodule-Like Outgrowths on cv Afghanistan Roots Contain PsENOD12 and PsENOD2 Transcripts

Having established that NPA induces PsENOD12 gene expression in root hairs, we investigated whether NPA at 10^{-5} M triggers the expression of the early nodulin genes PsENOD12 and PsENOD2 in pseudonodules as well. RNA transfer blot analysis revealed that both PsENOD12 and PsENOD2 mRNAs were detectable in NPA-induced pseudonodules, but at a lower level than in nodules formed in response to *R. leguminosarum* bv. *viciae* TOM (Fig. 2). A PsENOD12 transcript approximately 600 nucleotides long and a PsENOD2 transcript approximately 1500 nucleotides long were detected on transfer blots of RNA isolated from nodules of cv Afghanistan induced by *Rhizobium* or from NPA-elicited pseudonodules. Transcripts of the same size were detected previously in Rondo peas (25). No PsENOD12 or PsENOD2 transcripts were detected in uninoculated roots (Fig. 2). Therefore, the NPA-induced pseudonodules formed on roots of cv Afghanistan contain some of the same nodulin mRNAs as *Rhizobium*-elicited nodules.

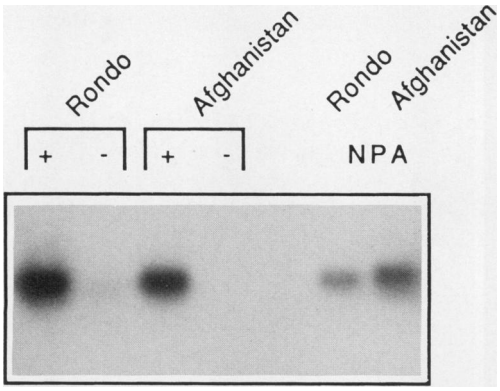

Figure 1. Polymerase chain reaction analysis of PsENOD12 gene expression in root hair RNA. The different lanes contain amplified root hair PsENOD12 sequences from uninoculated pea plants (Rondo−, Afghanistan−), from plants inoculated with *R. leguminosarum* bv. *viciae* (Rondo+, Afghanistan+), and from plants treated with 10^{-5} M NPA (Rondo NPA, Afghanistan NPA).

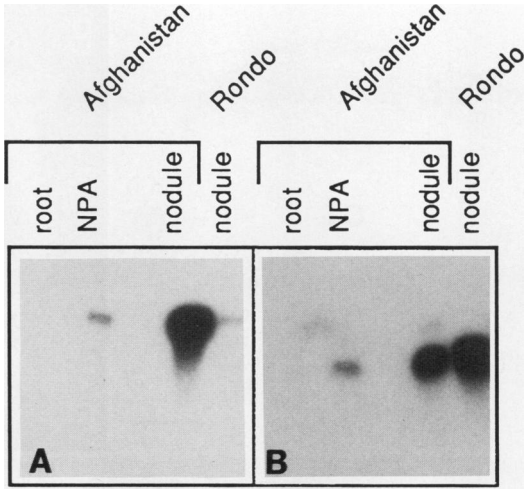

Figure 2. Autoradiographs of an RNA transfer blot probed with a radioactively labeled cDNA clone of either A, PsENOD2 or B, PsENOD12. The lanes contain RNA from uninoculated roots, pseudonodules induced by NPA, and nitrogen-fixing nodules formed on cv Afghanistan, as well as nodule RNA from cv Rondo. The PsENOD2 mRNA is approximately 1500 nucleotides long, whereas the PsENOD12 mRNA is 600 nucleotides long. The PsENOD2 mRNA signal in Rondo nodules is not very strong probably because very young nodules were collected for this assay.

Histology of Nodule-Like Structures Induced by NPA on *P. sativum* cv Afghanistan Roots and in Situ Localization of PsENOD2 and PsENOD12 mRNA

Nitrogen-fixing nodules induced by *R. leguminosarum* bv. *viciae* TOM on roots of cv Afghanistan are typical indeterminate nodules with a meristem at the distal end. Just proximal to the meristem is the invasion zone, and adjacent to the invasion zone are the following: the early symbiotic zone (the distal region of the infected cell zone), the late symbiotic or infected cell zone, and the senescent zone. The central tissue consisting of infected and uninfected host cells is surrounded by a region of cells known as nodule parenchyma ("inner cortex"), which is separated from the nodule cortex by the nodule endodermis (Fig. 3A).

In *R. leguminosarum* bv. *viciae* TOM-induced nodules, PsENOD2 mRNA was specifically localized by in situ hybridization to the nodule parenchyma (Fig. 3, A and B), whereas PsENOD12 mRNA was detected in the invasion

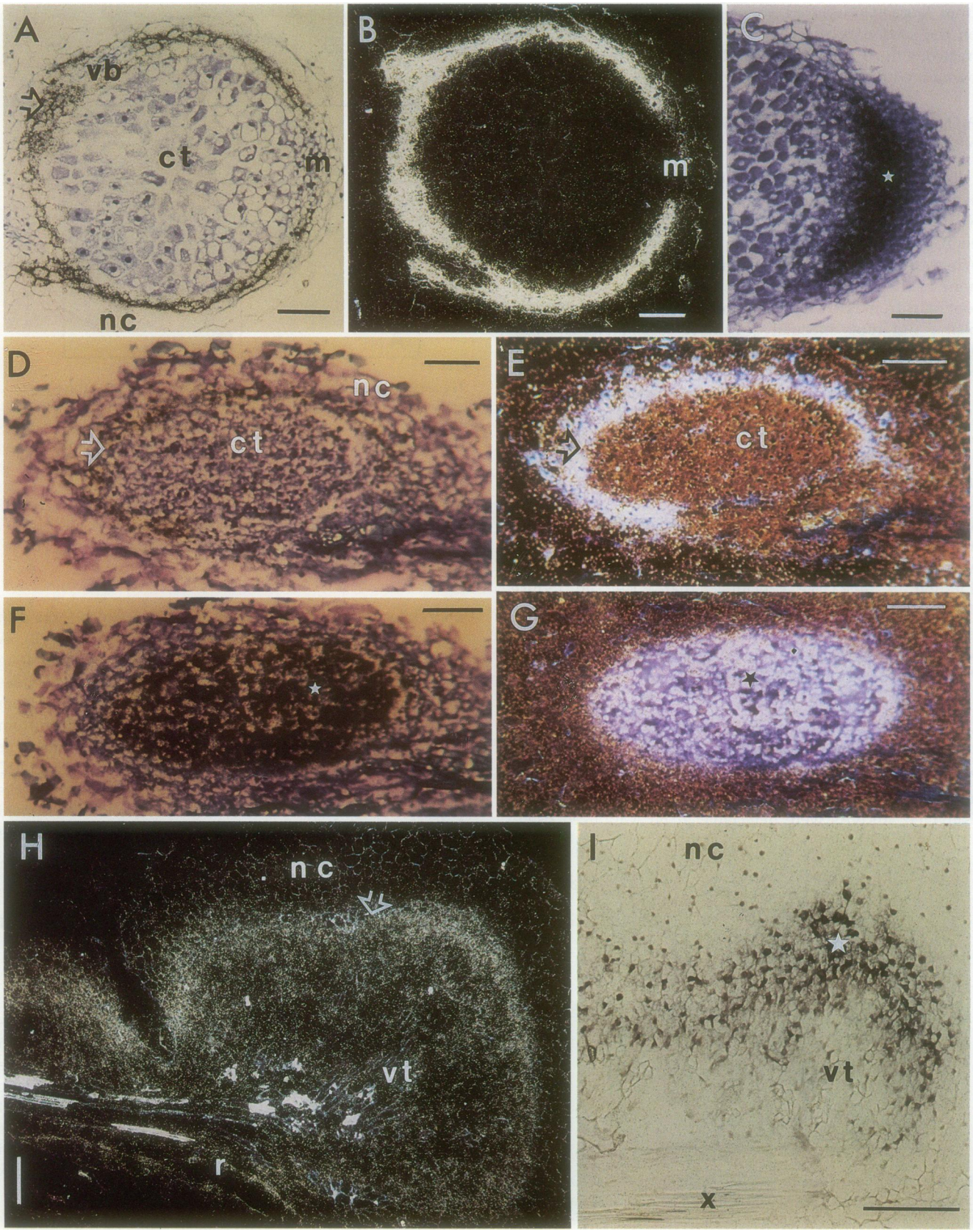

Figure 3. A, Longitudinal section of a nitrogen-fixing nodule on a root of cv Afghanistan. The section was hybridized with a [^{35}S]UTP-labeled antisense probe of PsENOD2. PsENOD2 transcripts, here seen as dark grains, are localized to the nodule parenchyma (arrow), which is internal to the nodule endodermis and surrounds the vascular bundle (vb). No transcripts are detected in the nodule meristem (m) or in the

zone proximal to the apical meristem (* in Fig. 3C). Hence, the location of PsENOD2 and PsENOD12 transcripts in nitrogen-fixing nodules of cv Afghanistan is identical to that described previously for cv Rondo (25, 31).

The structure of the pseudonodules induced by NPA on cv Afghanistan varied considerably; three major size classes were observed. These size classes most likely represent different developmental stages of the same structure. This assumption could not be tested, however, because of the nonsynchronous response of the plants to NPA treatment.

The smallest pseudonodules (<1 mm) contained small but highly vacuolate cells throughout the outgrowth. The central cells were smaller than the outer cells, which resembled a nodule cortex (Fig. 3D). No conspicuous tracheids or other vascular elements were observed in the central tissue. In these pseudonodules, we found that a narrow zone consisting of cells of small diameter and surrounding the central tissue contained PsENOD2 transcripts (Fig. 3, D and E). The surrounding, highly vacuolated cells that resemble a nodule cortex did not contain transcripts that hybridized to the PsENOD2 probe. We hybridized sections immediately adjacent to those shown in Figure 3, D and E, with the PsENOD12 antisense probe and found that PsENOD12 transcripts were specifically localized to the central tissue (Fig. 3, F and G). From these analyses, we concluded that a PsENOD2-expressing layer surrounds a central tissue in which PsENOD12 transcripts are found.

Intermediate-sized (1–3 mm) nodule-like structures were also found on roots of cv Afghanistan. In these pseudonodules, tracheary elements were present in the central tissue (Fig. 3H). Small-diameter, cytoplasmically rich cells surrounded the centrally located vascular tissue as well as cells thought to be derived from the pericycle (1). We detected PsENOD2 transcripts in the small-diameter cells located to the inside of the nodule endodermis. Because it is suberized, the nodule endodermis, which delimits the PsENOD2-expressing region from the nodule cortex, can be observed as a layer of cells that reflects light when viewed under dark field (Fig. 3H). PsENOD12 transcripts were detected just internal to the PsENOD2-expressing cells in the presumed pericycle derivatives. These cells are distal to the most recently differentiated cells of the vascular tissue (Fig. 3I). No early nodulin mRNAs were detected in the central, vascularized region of the pseudonodules.

The largest root outgrowths (>3 mm) resembled the hypertrophies formed on soybean and cowpea roots after treatment with the ATI, 2-bromo-3,5-dichlorobenzoic acid (1). These large pseudonodules were characterized by a central region of vascular tissue that contained conspicuous tracheary elements as well as cells presumably derived from the pericycle (1). The central tissue was delimited from a nodule cortex-like tissue of highly vacuolate cells by a narrow band of small-diameter cells rich in cytoplasm. An endodermis was positioned externally to this zone of cytoplasmically rich cells. Even though we examined more than 15 outgrowths of the largest nodule-like structures elicited by NPA treatment by in situ hybridization, we found that PsENOD2 or PsENOD12 transcript levels were often not above background (data not shown).

DISCUSSION

During *Rhizobium*-induced nodule formation, PsENOD12 transcripts are found in two spatially distinct areas: in root hair and adjacent cells of the outer root cortex preparing for and containing growing infection threads, and in inner cortical cells that have recently divided and established the nodule primordium. The nodule primordium is displaced from the cells containing infection threads by three to six cortical cells. The fact that the PsENOD12 gene product is detected in both areas as the nodule develops suggests that PsENOD12 is important for both the infection process and nodule primordium formation. Based on the presence of proline-rich motifs in the putative protein (25), PsENOD12 is presumed to be a cell wall protein. The localization of PsENOD12 mRNAs to infected cells suggested that *Rhizobium* penetration of host cells triggers PsENOD12 gene expression. The PsENOD12 protein may be a component of the newly synthesized infection thread or of the wall of the infected cell itself. However, coincidentally, PsENOD12 transcripts are detected in a disparate location, the nodule primordium, making it difficult to determine in wild-type *Rhizobium*-induced nodules whether infection thread formation is a prerequisite for PsENOD12 gene expression in this location. By treating pea roots with NPA, we were able to determine whether cellular divisions that produce a structure similar to a nodule primordium were sufficient for eliciting PsENOD12 gene expression.

nodule cortex (nc). Bright field optics. ×82. B, The same nodule under dark-field optics. PsENOD2 transcripts are indicated by the brightly reflecting grains over the nodule parenchyma. ×82. C, Longitudinal section of the distal end of a mature nitrogen-fixing nodule. The section was hybridized with a [^{35}S]UTP-labeled antisense probe of PsENOD12. PsENOD12 transcripts (*) are indicated by the dark deposits over the invasion zone of the nodule. The nodule meristem is distal to the invasion zone and stains heavily with toluidine blue. ×82. D, Bright-field photograph of a small (<1 mm) pseudonodule induced by NPA. Silver grains (arrow) indicating PsENOD2 transcripts are present in the cells surrounding the central tissue. Silver grains are not observed in the central tissue (ct) or in the nodule cortex (nc). ×94.5. E, Dark-field photograph of D. The silver grains (brightly reflecting region) surround the ct. The arrow points to the PsENOD2-hybridizing tissue. ×94.5. F, Bright-field photograph of the section adjacent to that pictured in D and E and hybridized with an antisense PsENOD12 probe. Silver grains are deposited over the central tissue (*). ×94.5. G, Dark-field photograph of F. ×94.5. H, Dark-field photograph of a midsized (1–3 mm) pseudonodule induced by NPA. PsENOD2 transcripts (brightly reflecting silver grains) are detected in the cells within the nc. The outer edge of this region is also brightly reflecting due to the suberized endodermal cells (arrow). The tissues comparable to nc and ct, which contains vascular tissue (vt), do not hybridize to the probe. r, Parent root. ×82. I, Bright-field photograph of a longitudinal section of a midsized pseudonodule induced by NPA. PsENOD12 transcripts (visualized by a purple color) were localized on paraffin sections using the Boehringer-Mannheim RNA labeling kit. The PsENOD12 hybridizing zone (*) is internal to cells that express PsENOD2. Cells comparable to a nc and cells of ct containing vt do not hybridize to the antisense PsENOD12 probe. x, Xylem of the parent root. ×164.

In this report, we found that the NPA-induced pseudonodules, which are devoid of infection threads, did contain PsENOD12 transcripts, indicating that infection per se is not a prerequisite for the induction of PsENOD12 gene expression in Afghanistan pea. As in alfalfa (14), NPA mimics a condition brought about normally by *Rhizobium* infection. However, in Afghanistan pea, not only PsENOD2 but also PsENOD12 genes are expressed. Moreover, PsENOD12 is expressed even in NPA-treated root hairs of Rondo pea, a cv that does not form pseudonodules in response to NPA. We did not examine root hairs from cvs of pea other than cvs Rondo and Afghanistan. We do not understand why cv Afghanistan but not the other pea cvs tested responded to NPA treatment by forming pseudonodules. Experiments on Afghanistan × Sparkle hybrids indicate that the responsiveness of cv Afghanistan to NPA is not related to the presence of the *sym2* allele (A.M. Hirsch, unpublished results). The *sym2* allele enables cv Afghanistan to be nodulated by *R. leguminosarum* bv. *viciae* TOM, but not by "conventional" strains of *R. leguminosarum* bv. *viciae* such as strain 248 (15).

Besides NPA, the only other treatments that elicit PsENOD12 gene expression in root hairs and the central tissue of the developing nodule are infection with *Rhizobium* cells or incubation with Nod factor (23, B. Horvath, unpublished results). At least two possible explanations may account for our results: (a) NPA mimics the Nod factors by binding to the same receptor, or (b) NPA initiates a series of responses that are downstream in the signal transduction pathway induced by the Nod factor. The structural dissimilarity between the two molecules and the fact that the ATIs do not cause root hair deformation argues against the first possibility. Furthermore, most pea cvs develop nodules in response to *Rhizobium* and not to NPA, implying that NPA and *Rhizobium* differ in their initial mode of action. Thus, we favor the second explanation.

Although the overall morphology of the NPA-induced pseudonodules differs from that of *Rhizobium*-induced Afghanistan pea nodules, the spatial distribution of both the PsENOD2 and PsENOD12 transcripts in the pseudonodules is reminiscent of their distribution in normal nitrogen-fixing nodules. The following differences, however, were observed. In *Rhizobium*-induced nodules, PsENOD2 transcripts were not detected in the nodule meristem; there was a gap of PsENOD2 transcript localization at the distal end of the nodule. However, no such gap in PsENOD2 expression was found in NPA-induced pseudonodules, most likely because they lack a meristem at their distal ends. Similarly, an apparent difference in the location of PsENOD12 gene expression was observed between normal nitrogen-fixing pea nodules and pseudonodules formed in response to NPA treatment. In *Rhizobium*-induced nodules, PsENOD12 is expressed in a region of cells proximal to the apical meristem. In NPA-induced pseudonodules, PsENOD12 transcripts are localized to cells that are adjacent and internal to the PsENOD2-expressing region. In the smallest pseudonodules examined, the PsENOD12-expressing cells constitute the central region of the nodule-like structure. In larger pseudonodules, the cells expressing PsENOD12 become restricted to a region just inside the PsENOD2-expressing zone and outside of the region containing the most recently differentiated tracheary elements; the central region is occupied by mature vascular tissue.

We propose that the PsENOD12-expressing zone in the small, NPA-generated pseudonodules is equivalent to the nodule primordium found in the earliest stage of development of a *Rhizobium*-induced nodule. Like the nodule primordium, the PsENOD12-expressing zone of NPA-induced pseudonodules is physically separated from the root hairs and outer cortical cells and is surrounded by cells that contain PsENOD2 transcripts. In the intermediate-sized pseudonodules, the PsENOD12 transcript-containing zone is correlated with the invasion zone, which is internal to nodule parenchyma tissue (the ENOD2-expressing region) and proximal to the nodule meristem in *Rhizobium*-induced nodules (25, 31; cf. Fig. 3, C and I). The nodule primordium and the invasion zone most likely represent different developmental states of the same tissue. In pea, the nodule primordium becomes apparent as a nest of newly divided cells in the root inner cortex within a few days after inoculation with *R. leguminosarum* bv. *viciae*. The primordium becomes invaded by growing infection threads that have advanced inward from the root hair and outer cortical cells. Upon invading the nodule primordium, the infection threads reorient their direction of growth and extend toward a nodule meristem that is initiated on the distal end of the primordium (20). The change in position of ENOD12 gene expression from nodule primordium to invasion zone in pea is thus correlated with the outgrowth of the apical meristem and the continuous differentiation of cells expressing PsENOD12 adjacent to the meristem. However, the displacement in the localization of PsENOD12 gene expression from the entire center of the pseudonodule to a distal part of the central tissue in NPA-induced nodules cannot be correlated with meristem outgrowth. Rather, it probably is related to the differentiation of vascular tissue from the parent root into the pseudonodule.

Our results are consistent with the hypothesis that PsENOD12 as well as PsENOD2 are molecular markers for cells with altered hormone levels. NPA is presumed to perturb the endogenous hormone balance of the treated roots by binding to a protein localized on cellular membranes and inhibiting auxin transport (16). In favor of the hypothesis that hormones trigger early nodulin gene expression, Dehio and de Bruijn (5) found that treating roots of *Sesbania rostrata* with zeatin induced the expression of SrENOD2. NPA could conceivably lead to a hormone imbalance, e.g. an excess of cytokinin in the root, by blocking the basipetal movement of endogenous auxin.

However, why only certain root cells respond and how the growth hormone balance of the responding cells is changed, either following NPA treatment or inoculation with *Rhizobium*, remain unknown. A gradient of cell response has previously been observed in inoculated pea roots. Cells of the outer cortex realign their cytoplasms to form a "cytoplasmic bridge" 48 h after inoculation (17), whereas inner cortical cells divide and establish the nodule primordium, which usually develops opposite a protoxylem pole (20). Libbenga et al. (21) showed that a factor from the xylem stimulates cell division in the inner cortex of pea root explants in the presence of auxin and cytokinin. The presence of such a factor may make the inner cortical cells more competent

than other root cells to undergo cell division. However, both inner and outer cortical cells undergo extensive rearrangements of microtubules after inoculation with *Rhizobium* (17). Thus, the disparate localization of PsENOD12 gene expression may be related to cytoskeletal reorganization brought about by local changes in hormone balance. Upon inoculation with *Rhizobium*, Nod factors may alter the endogenous hormone balance at the infection site, whereas an unknown factor, presumably from the xylem, is involved with either producing or maintaining localized hormonal perturbations, which had been brought about by diffusible signals from *Rhizobium* or endogenous secondary signals, in the root inner cortical cells. In either case, PsENOD12 genes are expressed in these cells as a result.

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