Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors

(nodules/nodule-specific genes/development)

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ABSTRACT Rhizobium nod genes are essential for root hair deformation and cortical cell division, early stages in the development of nitrogen-fixing root nodules. Nod− mutants are unable to initiate nodules on legume roots. We observed that N-(1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid, compounds known to function as auxin transport inhibitors, induced nodule-like structures on alfalfa roots. The nodule-like structures (pseudonodules) were white, devoid of bacteria, and resembled nodules elicited by Rhizobium meliloti exopolysaccharide (exo) mutants at both the histological and molecular level. Two nodulin genes, ENOD2 and Nms-30, were expressed. RNA isolated from the nodule-like structures hybridized to pGmENOD2, a soybean early nodulin cDNA clone. RNA isolated from roots did not hybridize. We determined by in vitro translations of total RNA that the alfalfa nodulin transcript Nms-30 was also expressed in the nodule-like structures. The late expressed nodulin genes, such as the leghemoglobin genes, were not transcribed. Because N-(1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid induce the development of nodules on alfalfa roots, we suggest that the auxin transport inhibitors mimic the activity of compound(s) made upon the induction of the Rhizobium nod genes.

The nodulation (nod) genes of Rhizobium play an essential role in the induction of nodules on the roots of leguminous plants. The importance of the nod genes has been demonstrated in at least two ways. First, it has been shown that Rhizobium that have mutated common nod genes, nodABC, lose the ability to curl root hairs (1) as well as to initiate cortical cell divisions within the root cortex. Cortical cell divisions mark the beginning of root nodule formation (2). Second, by transferring the nodule region of Rhizobium meliloti or Rhizobium leguminosarum to Agrobacterium tumefaciens, Agrobacterium transconjugants acquire the ability to form nodules on alfalfa and Vicia, respectively (3, 4).

Although the nod genes are required for the earliest stages of nodule development, it is not known how the nod gene products induce root hair curling and cortical cell divisions. The gene products of nodA and nodC have been immunolocalized in the rhizobial cytosol and outer membrane, respectively (5, 6). John et al. (7) have proposed that the nodD gene product aids in the transfer of growth factors from Rhizobium to the plant. Studies of nod gene regulation (cf. publications in ref. 8) have indicated that flavones—e.g., luteolin, apigenin, and 7,4′-dihydroxyflavone—are essential for the expression of nod genes in Rhizobium (9–11). However, the function(s) of the nod gene products has yet to be determined.

Plant hormones have been suggested as playing a critical role in nodule development since the first report of auxin involvement in pea root nodulation by Thimann (12). Rhizobium produces auxins, cytokinins, and gibberellin-like substances (cf. reviews in refs. 13 and 14), but none of the common nod genes has been shown to be involved in plant hormone synthesis. Libbenga et al. (15) studied the initiation of cortical cell divisions in legume root tissues in response to auxins and cytokinins in an effort to relate hormone levels to nodule formation. Bauer et al. (16) demonstrated that the cytokinin benzyladenine, as well as unknown diffusible substances produced by homologous but not heterologous rhizobia, induced cortical cell divisions in a number of legumes. Despite these efforts, a precise role for the plant hormones, whether endogenous or introduced by the bacterial symbionts, in nodule initiation and development remains undefined.

There are reports on the formation of nodule-like structures or pseudonodules on roots by treatment with the cytokinins (17, 18). However, the internal structure of these cytokinin-induced pseudonodules differed from that of Rhizobium-induced nodules. Substituted benzoic acids, chemicals that presumably modify auxin levels in the plant (19, 20), also induce nodule-like structures on legume roots (refs. 21 and 22; J.G.T., unpublished results).

We found that the histology of the nodule-like structures induced by the auxin transport inhibitors N-(1-naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) closely resembled that of Rhizobium-induced legume nodules. We studied these nodule-like outgrowths to determine their relation to root nodules initiated by Rhizobium. In particular, we found that the pseudonodules were very similar to nodules induced by exopolysaccharide (exo) mutants of R. meliloti (23). R. meliloti exo mutants, although unable to penetrate alfalfa root tissue, are capable of inducing cortical cell divisions and nodule development. Apparently, the Rhizobium mutants produce a signal, which is expressed within the root cortex, at a distance removed from the source of the signal.

While more than 20 nodulins have been identified in wild-type-induced alfalfa nodules, only two nodulin genes have been shown to be expressed in nodules induced by exo mutant R. meliloti. They are the Nms-30 gene, identified by in vitro translation of nodule RNA, and a gene in alfalfa comparable to the soybean early nodulin gene ENOD2. Transcripts for other nodulin genes, such as the Lb genes, are not present in the exo mutant-induced nodules (24–26).

We used cloned early nodulin DNAs as probes to investigate gene expression of the nodule-like structures induced by NPA and TIBA. Our goal was to determine whether the pseudonodules elicited by the auxin transport inhibitors

Abbreviations: NPA, N-(1-naphthyl)phthalamic acid; TIBA, 2,3,5-triiodobenzoic acid.

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resembled *Rhizobium*-induced nodules at the molecular level.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions.** Seeds of *Medicago sativa* L. cv. Iroquois (alfalfa) were sterilized as described (27) and sown in autoclaved plastic pans containing sterilized perlite covered with 2 cm of sterile vermiculite. The pans were watered with 1.5 liters of Jensen’s medium (28) without nitrogen at the time of sowing. The seedlings were grown under conditions of 16 hr, 21°C day/8 hr, 19°C night. Two-week-old seedlings were watered with an additional 1.5 liters of Jensen’s medium containing dilutions of the various benzoic and phthalamic acid derivatives with 5–40 mM nitrate or without nitrate. Distilled water was used for watering the seedlings between and after treatments.

Additional plants were grown in test tubes as described by Meade et al. (27). Benzoic acids were applied as aqueous solutions to the seedling roots 2 weeks after planting.

Alfalfa seedlings were inoculated with wild-type or *exo* mutant *R. meliloti* as described (25).

**Benzoic and Phthalamic Acid Derivatives.** TIBA (Sigma) was dissolved in ethanol and diluted to $5 \times 10^{-5}$ M in Jensen’s medium containing nitrate. A $10^{-2}$ M stock solution of NPA [2-(1-naphthalenylaminocarbonyl)benzoic acid] (U.S. Rubber) was prepared in an alkaline aqueous solution and diluted to $10^{-5}$ M before application to plants.

**RNA Isolation.** Roots and their nodule-like structures were harvested separately 3–4 weeks after treatment, frozen immediately in liquid nitrogen, and stored at $-70^\circ$C. RNA was isolated separately from the frozen roots and nodule-like structures as described (29).

**In Vitro Translations and RNA Transfer Blots.** Total RNA was translated in a rabbit reticulocyte system (29) or prepared for RNA transfer blots as described by Maniatis et al. (30). The RNA for transfer blots was transferred to GeneScreen (New England Nuclear) according to the manufacturer’s directions. A 1065-base-pair insert of pENOD2 (31) was prepared and nick-translated. Hybridization and washing conditions followed the GeneScreen manufacturer’s protocol, washing at either 42°C or 65°C.

**Microscopy.** Excised nodule-like structures and roots were prepared for light microscopy using plastic sections (32).

**Recovery of Bacteria from Nodules.** Nodule-like structures were surface sterilized in 20% sodium hypochlorite for 2–3 min and washed three times, 5 min each, with sterile distilled water. The nodule-like structures were squashed whole in a minimal volume of sterile water and dilutions were plated on Luria broth agar without drugs. Recovered bacteria were replica-plated onto selective media for strain confirmation. Bacteria were also tested for nodulation ability following inoculation of axenically grown alfalfa seedlings.

**RESULTS**

**Formation of the Pseudonodules.** Structures resembling nodules were observed to develop on primary and secondary roots of alfalfa plants 2 weeks after applying either $5 \times 10^{-5}$ M TIBA or $10^{-5}$ M NPA. The maximum number of pseudonodules appeared 3–4 weeks after the compounds were applied (Fig. 1). The pseudonodules were harvested at this time. NPA was more effective than TIBA; 60% versus 25% of the plants formed nodules. Bacteria recovered from selective media and tested on alfalfa seedlings did not induce nodules.

Nitrates did not inhibit pseudonodule formation in the presence of either TIBA or NPA. In many of the plants, the presence of the auxin transport inhibitors resulted in an increase in diameter of the elongation region of the primary root. These roots superficially resembled the thick short root (*tsr*) response described for *Vicia sativa* roots (33).

**Histology.** The anatomy of the NPA- and TIBA-induced pseudonodules was similar to that described for “empty” alfalfa nodules—i.e., nodules elicited by the *exo* mutants of *R. meliloti* (23). This was also true for nodules induced by *Agrobacterium* transconjugants containing *R. meliloti* nodulation sequences (3). A meristem was present (Fig. 2), but it was more diffuse than the meristem of the nodules induced by wild-type *R. meliloti*. Nodule cortex cells and cells of the central tissue were clearly distinguishable from each other, many of the central cells being filled with amyloplasts (Fig. 2). Distinct endodermal formation and vascular tissue differentiation were evident only in the proximal part of the pseudonodules. In this respect, the pseudonodules differed from the *exo* mutant and *Agrobacterium* transconjugant-induced nodules. In the latter, vascular tissue differentiates into the distal part of the nodule.

**Gene Expression.** We analyzed RNA isolated from the pseudonodules for the presence of ENOD2 and Nms-30 transcripts to see if the nodule-like structures were similar at the molecular level to the empty nodules induced by *exo* mutant *Rhizobium*. The expression of the Nms-30 nodulin gene, previously described as N-38 (25, 26), was determined by *in vitro* translation of nodule RNA followed by two-dimensional gel electrophoresis. The alfalfa ENOD2 gene expression was studied by RNA transfer blot analysis using the soybean pGmENOD2 clone as a probe (31).

The nodule-like structures elicited by either TIBA or NPA expressed the ENOD2 gene as did nodules induced by *exoH* or *exoB* mutants of *R. meliloti* (Fig. 3). The ENOD2 mes-
senger RNA was not found in RNA isolated from the tsr-like elongation zone of roots that were grown in medium containing either TIBA or NPA (Fig. 3).

Total RNA from pseudonodules of TIBA-treated plants was compared to RNA isolated from roots of untreated plants, wild-type nodules, and mutant exoB-induced nodules by in vitro translation and two-dimensional gel electropho-

resis of the translation products. Fig. 4 B–D shows that the messenger RNA for Nms-30 was present in wild-type and exoB mutant-induced nodules and also in TIBA-elicited nodule-like structures (indicated by arrowheads). No translation product of comparable size and charge was found among the translation products of root RNA (Fig. 4A; position marked by a circle). Other nodulins, such as Lb (indicated by arrows in Fig. 4B), were present only in the wild-type-induced nodules.

**DISCUSSION**

We have found that nodule-like structures initiated by auxin transport inhibitors on alfalfa roots are structurally similar to root nodules induced by *Rhizobium* exopolysaccharide mutants. These results confirm and extend earlier reports on the same subject (refs. 21 and 22; J.G.T., unpublished results). We have also determined that in these nodule-like structures two genes, ENOD2 and Nms-30, are expressed. Transcripts for these genes have previously been found to be present only in *Rhizobium*-induced root nodules.

Previously, we used histological criteria to describe the empty nodules induced by *Rhizobium* exo mutants and *Agrobacterium* transconjugants carrying the *nod* genes of *rhizobia* (3, 23). The nodule-like outgrowths initiated by the
two growth regulators, TIBA and NPA, resembled these empty nodules in histological organization, with one major exception. We did not observe differentiated vascular tissue in the distal portion of the nodule-like structures.

A characteristic feature of nodules induced by rhizobia is expression of genes known as the nodulin genes. Some of these are involved in nodule formation and others in nitrogen fixation. We believe that the expression of the two nodulin genes, ENOD2 and Nms-30, can serve as valid criteria to distinguish the chemical-induced outgrowths as nodules for the following reasons. The early nodule ENOD2 is expressed in all legume nodules studied to date (34). In addition, in situ hybridization studies show that ENOD2 is expressed exclusively in the inner cortex of the nodule in both determinate (soybean) and indeterminate (pea, alfalfa) nodules (C. van de Wiel and T.V.B., unpublished results; A.M.H., unpublished results). In these studies, messenger RNAs for ENOD2 were not found in the nodule meristem. This suggests that the expression of the ENOD2 gene reflects the differentiation of one of the tissues formed in true nodules. The early nodulin, Nms-30, is expressed abundantly in the wild-type and exo mutant-derived nodules of alfalfa. The role of Nms-30 in nodule development, however, is not clear. The expression of the ENOD2 and Nms-30 genes, as well as the histology of the root outgrowths, indicates that these nodule-like structures are indeed nodules.

The expression of the two nodulin genes in the absence of Rhizobium suggests that the two early nodulin genes may be developmentally, rather than symbiotically, regulated. However, expression in tissues other than nodules has not been demonstrated. The expression of the ENOD2 and Nms-30 genes is characteristic of empty alfalfa nodules (24–26). This suggests that Rhizobium is able to control the plant’s developmental program, resulting in the expression of nodulin genes. The expression of nodulin genes and the histological resemblance to Rhizobium-induced nodules leads us to speculate that Rhizobium may initiate nodule development by changing the cytokinin/auxin ratio of root cortical cells. This then causes cellular division. Rhizobium is known to secrete cytokinins. One possibility is that the nod genes encode products that directly or indirectly influence cytokinin secretion. This secretion would perturb the plant’s endogenous cytokinin/auxin ratio. Experiments by J. B. Cooper and S. R. Long (personal communication) support this interpretation. They observed that spot-inoculating alfalfa roots with an NODA mutant of R. meliloti, carrying the tzs (trans-cinnamoyl synthase or secretion; ref. 35) gene of A. tumefaciens, results in the formation of empty nodules not infected by Rhizobium. However, it is not known whether the nodulin genes, ENOD2 and Nms-30, are expressed in the nodules induced by these transconjugants.

Another possibility is that Rhizobium controls the auxin/cytokinin ratio by producing inhibitors of auxin synthesis, transport, or action. Jacobs and Rubery (36) reported that various plant flavonoids—e.g., apigenin—compete with synthetic auxin transport inhibitors. It is well documented that flavones regulate nod gene activity (9–11). It is feasible that Rhizobium synthesizes product(s), perhaps modified flavones, that function as auxin transport inhibitors.

The initial stages of nodule formation, root hair deformation and the induction of cortical cell divisions, are mediated by wild-type nod genes. We have shown that NPA and TIBA, two auxin transport inhibitors, induce cortical cell divisions and the development of nodules, which in turn express two nodulin genes. Thus, TIBA and NPA are able to substitute for the activity of compounds made following the induction of Rhizobium nod genes.

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