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Expression of early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and *Rhizobium*-induced nodules may be conserved

(cytokinins)

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ABSTRACT **Transcripts for two genes expressed early in alfalfa nodule development (*MsENOD40* and *MsENOD2*) are found in mycorrhizal roots, but not in noncolonized roots or in roots infected with the fungal pathogen *Rhizoctonia solani*. These same two early nodulin genes are expressed in uninoculated roots upon application of the cytokinin 6-benzylaminopurine. Correlated with the expression of the two early nodulin genes, we found that mycorrhizal roots contain higher levels of *trans*-zeatin riboside than nonmycorrhizal roots. These data suggest that there may be conservation of signal transduction pathways between the two symbioses—nitrogen-fixing nodules and phosphate-acquiring mycorrhizae.**

Of the two commonly described symbioses of roots—nitrogen-fixing nodules in response to members of Rhizobiaceae or Frankiaceae and phosphate-acquiring arbuscular mycorrhizae (AM) between roots and endophytic fungi—the more ancient of the two is AM. Structures identified as arbuscules have been found in *Aglaophyton major*, an early Devonian land plant, providing evidence for at least a >400-million-year-old association between terrestrial plants and fungi (1). In contrast, the *Rhizobium*–legume symbiosis is much younger, having been established no more than 65 to 136 million years ago, when the angiosperms evolved. However, unlike the AM symbiosis, which is found in almost all angiosperm families, *Rhizobium*-induced nitrogen-fixing symbioses appear to be restricted to a single subclade of a larger nitrogen-fixing clade (2).

LaRue and Weeden (3) proposed that nodulation evolved from the more ancient AM association because of certain similarities between the two symbioses. For example, some legumes are Nod^- (absence of nodule formation) as well as Myc^- (absence of mycorrhizal colonization) (4, 5). In 21 of 45 Nod^- mutants of pea and vetch, diallelic crosses have shown that Myc^- and Nod^- cannot be uncoupled, demonstrating that both phenotypes are derived from mutations in the same gene. Myc^- is recessive, and in pea, there are at least five separate loci involved with AM formation (6). Another similarity is that the rhizobial signal molecule (Nod factor), which is composed of β-1,4-*N*-acetyl-D-glucosamine residues with a $\geq$C16 fatty acid on the nonreducing end of the molecule and various other substituents such as sulfate, fucose, etc. on the reducing end (see refs. 7 and 8), is more characteristic of fungi than bacteria. Chitin-like molecules are rarely found in bacterial cell walls, especially in the walls of Gram-negative bacteria. Interestingly, Nod factor by itself elicits molecular responses in the plant, including rapid root hair membrane depolarization (9, 10), increased Ca^{2+} levels (11), and the induction of plant genes known as early nodulin genes (12). In addition, numerous cytological responses such as root hair deformation and cortical cell division occur. Perhaps free-living nitrogen-fixing bacteria evolved the capacity to make a chitin-like or other masking "molecule" to keep them from triggering a host defense response when they invaded the plant roots (3). In support of this, Mithöfer *et al.* (13) reported that the β-glucan-induced plant defense response in soybean is suppressed by cyclic 1,3–1,6-β-glucans from *Bradyrhizobium japonicum*.

In this report, we examined alfalfa roots colonized by AM fungi for transcripts of two early nodulin (*ENOD*) genes, *MsENOD2* and *MsENOD40*. Early nodulin genes are characteristically expressed during nodule morphogenesis (12). The *MsENOD2* gene product is proposed to be a proline-rich protein on the basis of its nucleic acid sequence (14). In contrast, *MsENOD40* appears to have no open reading frames longer than 27 amino acids that start with a methionine and thus has been proposed to function as an RNA (15, 16). However, a recent report indicates that a small peptide can be translated from the *GmENOD40* sequence, and also that *ENOD40* sequences are present in a nonlegume, namely tobacco (17).

In this report, we show that mycorrhizae resemble nodules at the molecular level in that these two early nodulin genes are expressed. We also show that cytokinin induces both *MsENOD2* and *MsENOD40* expression in uninoculated roots, indicating this plant hormone may be part of the mechanism of signal transduction. To confirm this, we show that cytokinin, specifically *trans*-zeatin riboside (ZR), accumulates to higher levels in mycorrhizae as compared with nonmycorrhizal roots.

MATERIALS AND METHODS

Plant Cultivars, Fungus, and Growth Conditions. Alfalfa (*Medicago sativa* L. cv. Gilboa) seedlings were grown in sand under microbiologically controlled *Rhizobium*-free conditions as described previously (18) with the following modifications. Experiments were carried in 225-ml polyvinyl chloride cones (25 cm high and 5 cm in diameter). At planting, 12 pregerminated alfalfa seedlings were transferred to each cone as described previously (18). All plants received 8 mM NH_4NO_3 and 0.2 mM KH_2PO_4. Before planting, surface-sterilized spores of *Glomus intraradices* (Schenck and Smith) or *Rhizoctonia solani* were layered 4 cm or 8 cm, respectively, below the soil surface to supply 10 to 40 spores per seedling. To ensure that only AM fungi in the inoculum caused the reported responses, the control was prepared by applying an equal amount of autoclaved spores. The control treatment contained solid particles and spore extracts, but no

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Abbreviations: AM, arbuscular mycorrhizae; ZR, *trans*-zeatin riboside; BAP, 6-benzylaminopurine; DIG, digoxigenin; iPA, isopentenyladenine; IAC, immunoaffinity chromatography.

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viable AM fungal spores. In initial experiments, there was no mycorrhizal colonization of control plant roots.

At each harvest, four cones from each treatment were collected to serve as one replicate. At this time, we observed *R. solani* infection sites all along the root. The roots were immediately frozen in liquid nitrogen and kept at −70°C until assayed. Mycorrhizal colonization was estimated colorimetrically by measuring glucosamine released from fungal chitin (19). Experiments described in this report quantified changes in the apical portion (2–6 cm) of the roots. All assays were conducted in two or three replicates, each replicate containing roots from 12 seedlings. Analysis of variance was used to test for significant ($P \leq 0.05$) differences, and when appropriate, standard error (SE) values were calculated.

Cytokinin and Nod Factor Treatments of Uninoculated Roots. Three-day-old alfalfa (*M. sativa* cv. Iroquois) seedlings for the cytokinin and Nod factor experiments were transferred to autoclaved Magenta jars containing fresh Jensen's medium minus nitrogen plus purified Nod factor (PNF) or 6-benzylaminopurine (BAP). PNF [RmIVS(Ac)] was used at 10^{-8} M, whereas BAP was used at 10^{-6} M; we had previously determined that this was an optimal concentration for BAP (20). No fewer than three separate experiments were performed so that different batches of RNA would be examined to check for consistency of the response.

All plants were grown in a Conviron growth cabinet, where they were maintained under 16-h 21°C day/8-h 19°C night with a relative humidity of 80% and an irradiance of 315 mE $s^{-1} \cdot m^{-2}$ [1 einstein (E) = 1 mol of photons]. The bottoms of the Magenta jars were covered with aluminum foil to exclude light and the lids were not tightly sealed to avoid ethylene accumulation. Tissue was harvested into liquid nitrogen at the various time points and stored at −70°C until RNA was isolated.

RNA Isolation, RNA Transfer Blot Analysis, and *in Situ* Hybridization. Two different RNA extraction procedures were used. In one, total RNA was extracted from 0.3 g of roots, which had been frozen with liquid nitrogen and ground to a fine powder by using a mortar and pestle. The powder was then thawed and further ground in 820 μl of grinding buffer (8 M guanidinium hydrochloride/20 mM Mes, pH 3.2/20 mM Na_2EDTA/50 mM 2-mercaptoethanol) and 650 μl of acid phenol/chloroform/isoamyl alcohol (25:24:1 vol ratio). The mixture was transferred to a 2-ml Eppendorf tube, swirled vigorously on a Vortex mixer for 15 sec, and incubated at room temperature (25°C) for 2–20 min. Phase separation was done by centrifugation for 15 min (14,000 rpm) in an Eppendorf 5417C microcentrifuge at room temperature. Total RNA was precipitated from the upper phase by adding 1 vol of cold (−20°C) isopropyl alcohol, incubating at room temperature for 10 min, and centrifuging for 20 min (12,500 rpm) in a microcentrifuge at 4°C. The pellet was washed twice with 75% ethanol and air-dried. The RNA was resuspended in 60 μl of water by incubation at 60°C for 10 min and stored at −80°C until used. The second RNA extraction procedure was as previously described (21).

Samples (10 μg) of total RNA were separated by electrophoresis in Mops/formaldehyde/1.1% agarose gel and blotted on Hybond membrane (Amersham) (21). Some blots had 20 μg of total RNA loaded per lane for the RNA transfer blot analyses (21). The blots were hybridized with radioactively labeled [α-^{32}P]dCTP probes derived from *MsENOD40* (15) and *MsENOD2* (14) cDNA clones. An *Msc27* cDNA clone (22) was used to standardize the amount of RNA loaded. All probes were prepared using random primer DNA labeling. Blots were prehybridized for 4–6 h at 60°C in 5× Denhardt's solution/5× SSPE/0.5% SDS (1× SSPE = 150 mM sodium chloride/10 mM sodium phosphate, pH 7.4/1 mM EDTA). The hybridization was done in the same solution for another 24 h. At the end of the hybridization period, the membrane was washed once in 1× SSC/0.5% SDS for 10 min at room temperature, and then twice in 0.1× SSC/0.5% SDS at 55°C. The filters were then exposed to x-ray film (Fuji photo film, Japan or Bioworld, Dublin, OH) at −70°C for 1–10 days.

Mycorrhizae were harvested 18 days after germination, fixed in formalin/acetic acid/alcohol (FAA), and subsequently embedded in Paraplast (Fisher). The *in situ* hybridization procedures were performed as previously described (23) with the following modifications. The proteinase K (concentration increased to 5 μg/μl) step was performed at 37°C. The concentration of acetic anhydride was increased to 0.5%. Both nonradioactive and radioactive riboprobes made from *MsENOD40* and *MsENOD2* cDNAs were utilized. The slides were treated with 50 μg/ml RNase A after the washing steps. One additional blocking step was also included. The slides hybridized to radioactive probes were stained with toluidine blue (23) after the emulsions were developed. Micrographs were taken with Kodak Etkachrome tungsten 160 film on a Zeiss Axiophot microscope, scanned into a computer, and processed with Adode Photoshop.

Scanning Electron Microscopy. FAA-fixed tissue was dehydrated through alcohol and dried at the critical point in a Polaris critical point drying apparatus. The tissue was mounted on stubs, coated with gold/palladium, and examined with an ETEC Autoscan scanning electron microscope (Hayward, CA).

Southern Hybridizations and PCR. Total genomic DNA was extracted from spores of *G. intraradices* by following a previously published procedure (24). *Rhizobium meliloti* DNA was extracted according to Meade *et al.* (25). Both AM-fungal and *R. meliloti* DNA were digested with *Eco*RI and with *Pst*I. Thirteen to 15 μg of fungal DNA and 5 μg of rhizobial DNA were loaded per lane, then fractionated on a 1% agarose gel, and transferred to GeneScreen membrane as previously described (15). The blots were probed either with a digoxigenin (DIG)-labeled *nodC* fragment from *R. meliloti*, using the procedure described by Boehringer Mannheim, or with a ^{32}P-labeled *R. meliloti nodC* fragment, using standard procedures (26).

For polymerase chain reaction (PCR) experiments, two different sets of primers were used, each with a different G+C content. The first combination (called *nodC* primers) was 5′-CATCCCGGGSCCKTGYGCHATGTAYMG-3′ and 5′-ATGCTCGAGATGGCGRTCYTCACC-3′. The second set of primers (called CHI primers) had a lower G+C content and was similar to regions in chitin synthase that are conserved within *nodC* (27, 28). These primers consisted of 5′-CATCCCGGG-WKSYYTSTCMATGTATMG-3′ and 5′-ATGCTCGAGAA-WTCTATCTTCASC-3′. One microgram of total DNA isolated from *Bradyrhizobium* sp. RP501, AM-fungal spores, or yeast was used in a 100-μl reaction mixture. Primers were added to a concentration of 0.1 μM, and 2.5 units of *Taq* DNA polymerase was added. The PCR started with 3 min at 95°C, followed by 30 cycles of 95°C for 1 min, 1 min annealing, and 1 min at 72°C. The reaction ended with 10 min at 72°C. Annealing temperatures of 40°C, 50°C, and 55°C were tested. A 30-μl sample of the reaction mixture was subjected to electrophoresis on a 1.2% agarose gel.

Southern hybridization analysis was performed to verify that the DNA derived from *Glomus* spores was not contaminated by plant DNA. Probes were made with the insert of *A2ENOD2* (14) and also with the internal *Hin*dIII fragment of the *MsENOD40–2* (15) cDNA clone according to published procedures (26). The Southern blots were also hybridized with an ≈600-bp *Eco*RI–*Kpn*I fragment derived from pKEPho, a plasmid which contains the phosphate transporter gene of *Glomus versiforme* (29), to evaluate the fungal DNA.

Cytokinin (CK) Analysis. Plant material was ground in liquid nitrogen and 0.5 g of the ground material was extracted with 80% MeOH containing 0.02% sodium diethyldithiocarbamate. Extraction was performed in the dark overnight with continuous stirring at 4°C. After filtration, the extract was reduced to dryness by rotary evaporation at 40°C and brought up to 5 ml in 0.1 M sodium acetate buffer at pH 3.6. The extract solution was then loaded onto a polyvinylpyrrolidone column (3 ml bed volume) and cytokinins were eluted with 10 ml of the same buffer. The

eluent was adsorbed onto a prewetted Sep-Pak cartridge (Waters) and washed with 4 ml of distilled water, and the cytokinins were then eluted first with 4 ml of 25% (vol/vol) MeOH (for ZR-like cytokinins) and then with 75% MeOH [for isopentenyladenine (iPA)-like cytokinins]. After the samples had been concentrated to 1 ml, they were transferred to 1.5-ml polypropylene microtubes (Elkay, Elreann, Ireland) for quantification.

Radioimmunoassay (RIA). Cytokinins were quantified by RIA using polyclonal antibodies for ZR and iPA, prepared according to MacDonald *et al.* (30) with the following modifications. Three hundred microliters of phosphate-buffered saline (PBS; 50 mM sodium phosphate, pH 7.●/140 mM sodium chloride) containing 0.1% gelatin was added to each microtube and the cytokinins were dissolved by incubating for 1 h at 37°C. Then 50 μl of the [^{3}H]cytokinin trialcohol analog was added according to the retention time. After thorough mixing, 50 μl of the suitable antibodies was added and the solution was mixed again. Following incubation at 37°C for 60 min, the antibodies were precipitated by addition of 600 μl of saturated ammonium sulfate and centrifugation at 13,000 rpm for 2 min (Biofuge 13, Heraeus). After the supernatant had been removed, 50 μl of MeOH was added and the mixture was incubated for 10 min. Radioactivity was determined directly in the microtubes by adding 1 ml of scintillation liquid (Lumax, Groningen, The Netherlands).

To prevent possible misinterpretation of the results due to interfering compounds, all the RIAs were performed at three different dilutions. A linear correlation was found between the dilution and the cytokinin concentration.

Immunoaffinity Chromatography (IAC). To confirm the results obtained by RIA, we utilized IAC followed by HPLC and analysis of UV spectra as previously described (31).

Each aliquot was dissolved in PBS containing 1 mM Na_2EDTA (Triplex III) and adjusted to pH 7 with NaOH. The extract was filtered and taken through a precolumn containing 1 ml of Sepharose 6B (Pharmacia). The samples were chromatographed through an IAC column, essentially as described by Nicander *et al.* (32) except that the column and solution temperatures were 30–35°C and for the comparison of extraction methods, ethanol was used as the eluent. The eluted fraction was evaporated under vacuum to about 300 μl, diluted by the addition of PBS, and taken through the process a second time. The new eluted fractions were vacuum-evaporated, and aliquots were pooled as appropriate.

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of a 10 × 250 mm semipreparative RP-18 column (Rainin Instruments) and Pharmacia LKB grant pump model 2249. Cytokinins were separated with a 30-min linear gradient of 20–90% (vol/vol) MeOH (HPLC grade; Baker) in water containing 0.1 M acetic acid. The flow rate was 3 ml/min. For UV analysis, we used an on-line scanning spectrophotometric detector (Spectra-Focus, Spectra-Physics).

Peaks identified by UV were collected and analyzed by RIA as described earlier for ZR-like activity.

RESULTS

Expression of *ENOD* Genes. We first examined RNA isolated from alfalfa total root systems that had been inoculated 40 days earlier with *G. intraradices*, *R. solani*, or both, or left uninoculated. There was no difficulty in distinguishing roots that had been inoculated with the AM fungi or with *R. solani*; *Glomus* produces spores and *R. solani* forms sclerotia. Hybridization to *MsENOD40* or *MsENOD2* probes occurred only in the roots that had been inoculated with *G. intraradices* (Fig. 1). At 29 days after inoculation, we detected some *MsENOD40* transcript accumulation but little or no *MsENOD2* mRNA accumulation, most likely because of the dilution of *ENOD2* mRNA by total RNA. The extent of *ENOD* transcript accumulation in RNA isolated from 28-day-old nodules is shown in lane N (Fig. 1).

We then analyzed RNA from root segments 2–6 cm behind the root tips to determine how early the *ENOD* transcripts could be

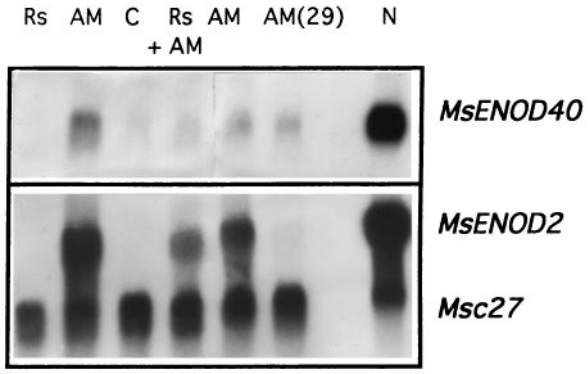

FIG. 1. Early nodulin (*MsENOD40* and *MsENOD2*) genes are expressed in roots colonized by AM fungi, but not in those infected by a pathogen only. Rs, *R. solani*; C, uninoculated control; Rs + AM, inoculated with both *R. solani* and AM fungi; AM(29), mycorrhizae 29 days after inoculation; N, nitrogen-fixing nodules.

detected. Fig. 2 shows that both *MsENOD40* and *MsENOD2* transcripts were found in mycorrhizal root RNA at the earliest time point. We were unable to determine whether there was a linear increase in transcript accumulation with time, however, because not all lanes were equally loaded (see Fig. 2 *Right* for rRNA levels). Overall, the level of *MsENOD40* transcript accumulation was significantly lower than that for *MsENOD2* at the various time points. No *ENOD* transcripts were detected in noncolonized or *R. solani*-infected roots at any time (Fig. 2).

To verify that the fungus did not express genes homologous to *MsENOD2* or *MsENOD40*, we probed DNA derived from spores with *MsENOD2* and *MsENOD40* cDNA clones. No *ENOD* gene homologs were found on the Southern blots (data not shown). In addition, DIG-labeled riboprobes did not hybridize to fungal cells in the *in situ* hybridization experiments either in mycorrhizal alfalfa roots (see Fig. 3G) or tobacco roots (data not shown). To verify that fungal DNA was present on the blot, we probed the same Southern blots with the insert of pKEPho; hybridization was observed (data not shown).

We then proceeded to determine the cell types in which the *ENOD* transcripts accumulated in the mycorrhizae. Fig. 3*A* illustrates the tree-like structure known as an arbuscule. These elaborations of the fungus are surrounded by a thin layer of plant cytoplasm bounded by plant membrane; reciprocal transport of metabolites is thought to occur at this junction (33).

MsENOD40 transcripts were localized to a number of cell types by *in situ* hybridization analysis. In radioactively probed sections allowed to expose for 6 weeks and then examined with dark-field optics, silver grains indicating the presence of *MsENOD40* mRNAs were detected mostly in the pericycle and around the periphery of the arbuscules in the cortical cells (Fig.

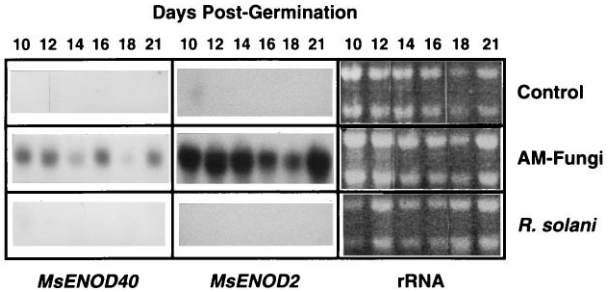

FIG. 2. RNA transfer blot showing *MsENOD40* and *MsENOD2* expression in control, mycorrhizal, and *R. solani*-infected roots 10–21 days post germination. Approximately 10 μg of RNA was loaded per lane. There were some fluctuations in the amount of RNA loaded in several lanes, as can be seen from the ethidium bromide-stained rRNA bands (rRNA lanes).

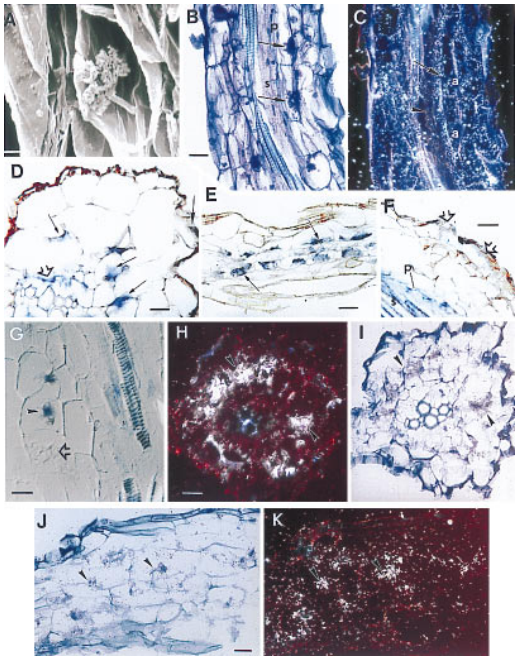

FIG. 3. (*A*) Scanning electron micrograph of an arbuscule in an alfalfa root. (Bar = 5 μm.) (*B*) Bright-field micrograph of a mycorrhizal root sectioned longitudinally. Arrows indicate arbuscules. The pericycle (p) and stele (s) are indicated. (Bar = 22 μm.) (*C*) Dark-field micrograph of *B* illustrating the presence of *MsENOD40* transcripts (shown here as white dots representing silver grains) in root cortical cells surround the arbuscules (a) and in the pericycle (arrow), but not in the central part of the stele (arrowhead); ^{35}S-labeled probe. Exposed for 6 weeks. (*D*) Bright-field micrograph of a transverse section of a young mycorrhizal root; arbuscules are not fully developed. Blue color indicating the presence of *MsENOD40* transcript is detected in the pericycle (open arrow), the inner cortical cells (small arrows), and an epidermal cell (arrow); DIG-labeled probe. (Bar = 22 μm.) (*E*) Off-median longitudinal section through the inner cortex of a root. Arbuscules (small arrows) are in the inner cortical cells, and the blue color indicating the presence of *MsENOD40* mRNAs is present in these cells; DIG-labeled probe. (Bar = 44 μm.) (*F*) Longitudinal section. Blue color is found in the epidermal cells (open arrows) as well as the pericycle (p) and stele (s); DIG-labeled probe. (Bar = 22 μm.) (*G*) Longitudinal section of a mycorrhizal root taken with Nomarski optics. Blue color indicating *MsENOD40* transcript localization is found in the stele and pericycle and in two infected cells (arrowhead). Note that the fully developed arbuscule (open arrow) does not show the blue color; DIG-labeled probe. (Bar = 22 μm.) (*H*) Dark-field micrograph of a transverse section of mycorrhizal root. Silver grains indicating *MsENOD2* mRNAs are clustered over the inner cortical cells, which contain arbuscules (arrows); ^{35}S-labeled probe. Exposed for 3 weeks. (Bar = 22 μm.) (*I*) Bright-field micrograph of *H*. Arbuscules (stained purple, arrowheads) are within inner cortical cells. (*J*) Bright-field micrograph of an off-median longitudinally sectioned mycorrhizal root. The arrowheads indicate the arbuscules. (Bar = 22 μm.) (*K*) Dark-field view of *J*. Silver grains indicating *MsENOD2* mRNAs are clustered over the arbuscules (arrows); ^{35}S-labeled probe. Exposed for 3 weeks.

3*C*; arrow). No signal over background was detected in the stele, indicating that the signal found over the cortical cells was unlikely to be an artifact. Moreover, in roots or regions of the root where there were no arbuscules, the silver grain density was comparable to background (data not shown). The bright-field view is depicted in Fig. 3*B*.

We subsequently utilized DIG, which is more sensitive than ^{35}S for detecting transcripts that are expressed at very low levels (cf. Fig. 2). *MsENOD40* transcripts as indicated by blue color were localized more specifically to the pericycle (Fig. 3 *D* and *F*) and also to infected cortical cells (small arrows, Fig. 3 *D* and *E*) and the epidermis (Fig. 3*F*). Fig. 3*G* shows a longitudinal section of a mycorrhizal root viewed under Nomarski optics. *MsENOD40* transcripts were detected in the pericycle and in cortical cells containing immature arbuscules (cf. Fig. 3 *D* and *E*). The DIG-labeled probe did not hybridize to RNA in the cytoplasm overlying a mature arbuscule (open arrow; Fig. 3*G*). In the *in situ* hybridization analysis, *MsENOD40* transcripts were found in both uninfected (epidermal and pericycle) and infected cortical (containing immature arbuscules) cells.

Using a ^{35}S-labeled riboprobe, we detected *MsENOD2* transcripts in inner cortical cells containing mature arbuscules (Fig. 3 *H* and *K*). DIG-labeled probes gave comparable results (data not shown). A mycorrhizal root is viewed in transection in Fig. 3 *H* and *I*, whereas Fig. 3 *J* and *K* are glancing sections through a longitudinally sectioned root. These sections were taken from a region in the root comparable to that in Fig. 3*A*.

Cytokinin Induces *ENOD* Gene Expression in Uninoculated Roots. Earlier research had demonstrated that purified Nod factor induced the expression of *ENOD40* in uninoculated vetch roots (see ref. 12). A similar response has been found for *Glycine soja* and alfalfa; *ENOD40* transcripts were detected by RNA transfer blot analysis as early as 6 h after treatment (ref. 34; data not shown). To test whether *ENOD* gene expression could be induced in uninoculated roots by cytokinin, 3-day-old alfalfa seedling roots were incubated in nitrogen-free Jensen's medium containing 10^{-6} M BAP for 4 days.

RNA transfer blot analysis showed that a transcript that hybridized to the *MsENOD40* cDNA-derived probe could be detected as early as 6 h after the start of the experiment (Fig. 4). In this particular experiment, there was an unexplained increase in *ENOD40* mRNA levels at 36 h and a decrease at 96 h. In contrast to *ENOD40*, transcripts hybridizing to the *ENOD2* probe could not be detected until 24 h after the start of BAP treatment and also increased more or less linearly.

These results suggested to us that either a Nod factor or a cytokinin-type signal molecule could serve as the inducer of *ENOD* gene expression in mycorrhizae. Thus, we thought it prudent to determine whether sequences homologous to the *Rhizobium nodC* gene existed in DNA isolated from fungal spores.

Southern and PCR Analysis. Using a fragment of *nodC* from *R. meliloti* as a probe, we were unable to detect any homology to AM-fungal DNA on three separate Southern blots. Degenerate primers made to *nodC* did not amplify any DNA fragments from AM-fungal DNA or from yeast DNA (data not shown). The *nodC* primers, however, amplified a 210-bp fragment of *Bradyrhizobium* sp. RP501 DNA (35) (data not shown).

When we utilized primers made to regions within *nodC* that resemble yeast chitin synthase (27, 28) in a PCR, no PCR products were generated either from yeast DNA or from AM fungal spore DNA at the lowest annealing temperature of 45°C. The CHI primers were also unable to amplify a PCR product from *Bradyrhizobium* sp. RP501 DNA. However, primers based more

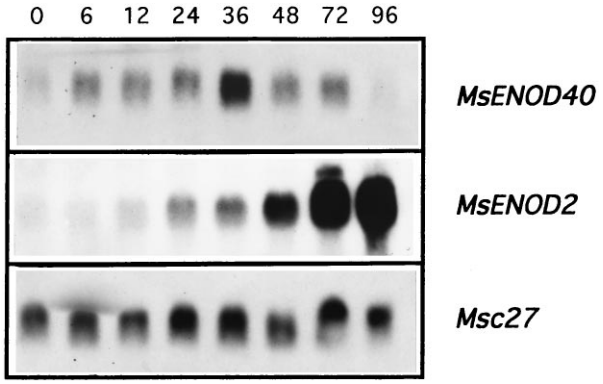

FIG. 4. BAP at 10^{-6} M induces *MsENOD40* or *MsENOD2* gene expression in uninoculated alfalfa roots harvested at the indicated time points (h). *Msc27* was used to standardize RNA levels.

closely on the yeast chitin synthase sequence amplified a PCR product from yeast (data not shown).

Cytokinin Evaluation. The effects of AM-fungal colonization on cytokinin levels were tested by two independent assays, IAC followed by HPLC analysis, and RIA. Cytokinins in mycorrhizal and nonmycorrhizal roots of 18-day-old alfalfa plants were purified by IAC and HPLC and were characterized by their UV spectra. ZR-type compounds (those that coeluted with standards for the nucleotide and free base forms of the Z-type cytokinins) were the most abundant cytokinins in the alfalfa roots, and their levels in mycorrhizal roots were significantly higher compared with nonmycorrhizal roots (Fig. 5). The RIA, which was carried out for the individually purified peaks, revealed a 3-fold increase in the mycorrhizal roots compared with the nonmycorrhizal roots for the two major peaks identified in the root extract. The mycorrhizal root extract showed an additional ZR-type compound that could not be detected in extracts from the nonmycorrhizal roots (Fig. 5). Levels of iPA were generally one order of magnitude lower than levels of the ZR-like compounds, and statistically significant differences could not be detected in the HPLC analysis (data not shown).

In the RIA experiments, the concentration of the ZR-type cytokinins was significantly higher in the mycorrhizal roots than in the control or *R. solani*-inoculated plants (Fig. 6). Similarly, coinoculation of AM fungi and *R. solani* together showed that ZR-type cytokinin concentration was higher in these roots than in the nonmycorrhizal controls or *R. solani*-only-infected roots (Fig. 6). Moreover, no difference in plant biomass or growth could be detected between the different treatments evaluated in this study.

DISCUSSION

We have shown that two early nodulin genes are expressed in roots colonized by AM fungi. We had anticipated that both *MsENOD2* and *MsENOD40* would be expressed in mycorrhizal roots because transcripts for these genes accumulate in the bacteria-free nodules elicited by *R. meliloti* exopolysaccharide (*exo*) mutants or in alfalfa roots treated with auxin transport inhibitors (15, 20, 36, 37). Although both early nodulin genes were expressed as early as 10 days after the start of the interaction with the AM fungi, the expression of neither gene was stimulated in roots infected with the fungal pathogen *R. solani*. This finding suggests that these are symbiosis-related genes and, moreover, that there are common elements between phosphate-acquiring mycorrhizae and nitrogen-fixing nodules.

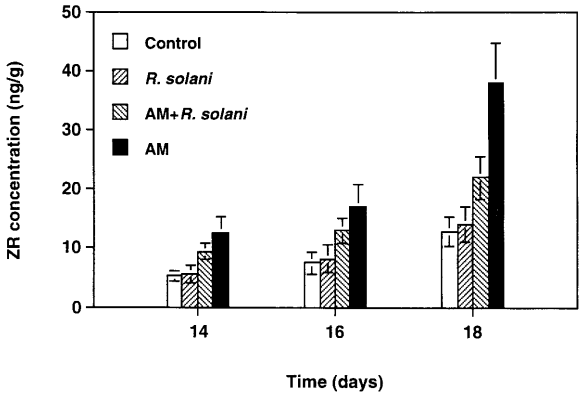

FIG. 6. Concentration of ZR (ng/g) over time in noncolonized roots, roots with AM, roots infected with *R. solani,* and roots with both *R. solani* and AM fungi.

As found for nodules, both colonized and noncolonized root cells contained *MsENOD40* mRNAs. Soon after rhizobial inoculation, *MsENOD40* transcripts were detected in epidermal, cortical, and pericycle cells as well as in the cells of the nodule primordia, which are derived from the inner cortex (refs. 15 and 16; Y. Fang and A. M. Hirsch, unpublished results). Similarly, in mycorrhizae, *MsENOD40* transcripts accumulated in the cells of the pericycle and to a more limited extent in the epidermis and inner cortical cells, especially those containing immature arbuscules.

MsENOD2 transcripts were detected in cells containing mature arbuscules. In nodules, *MsENOD2* transcripts accumulate in a peripheral tissue (the nodule parenchyma, formerly known as inner cortex) (37). Because of this location and its putative role as a proline-rich protein, the *MsENOD2* protein has been proposed to play a role in restricting oxygen to the central infected zone (38). There is no evidence for an oxygen barrier in mycorrhizae, so the *ENOD2* protein must

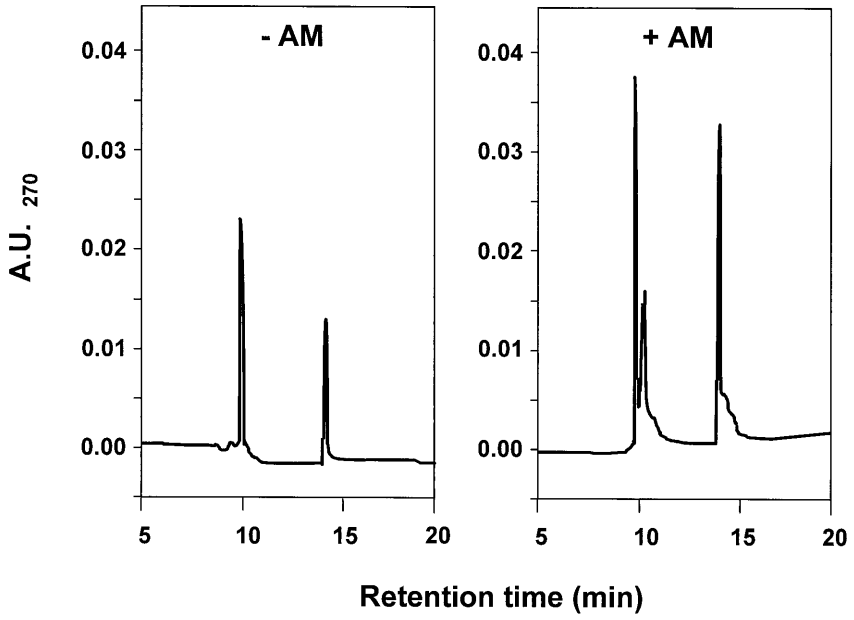

FIG. 5. HPLC chromatogram of IAC-purified extract of nonmycorrhizal (− AM) and mycorrhizal (+ AM) alfalfa roots. Samples (0.5 g dry weight) from 18-day-old plants were subjected to the extraction protocol described in the text. Peaks with retention times of 10 and 14 min correspond to the cytokinin standards, zeatin glucoside, and ZR, respectively.

have another function in this symbiosis. However, Frühling *et al.* (39) have determined that a novel leghemoglobin gene is induced in broad bean mycorrhizal roots; its function there is unknown. Bonfante-Fasolo *et al.* (40) have observed that hydroxyproline-rich glycoproteins are found at the interface between the fungus and its host, and Gianinazzi-Pearson (41) reported that *ENOD12*, which encodes another putative proline-rich protein, is expressed in mycorrhizal roots in cells containing arbuscules.

The results presented here are consistent with the hypothesis that signal transduction pathways between *Rhizobium* and AM fungi are conserved. However, so far there is no definitive evidence to suggest that this signal transduction pathway is mediated through a Nod factor-like molecule. Purified Nod factor induces *MsENOD40* gene expression, but not that of *MsENOD2* (data not shown), yet both are induced in mycorrhizal roots. However, Minami *et al.* (34) found that a mixture of Nod factors induced *GmENOD2* expression. The lack of amplification or hybridization to *nodC* as well as the lack of a PCR product following amplification with *nodC* primers suggests the signaling between plant and mycorrhizal fungi may not involve chitin-like molecules. Nevertheless, the argument could be made that there is not enough DNA sequence similarity across Prokaryota and Eukaryota to detect *nodC* homologs in AM fungi by Southern hybridization or by PCR. *N*-Acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-mannose, and sialic acid have all been localized to the cytoplasm between the arbuscule and the host plasma membrane (42), so it is likely that these molecules are important to the symbiosis.

The possibility exists, however, that phytohormones, specifically a ZR-type cytokinin, serve as the trigger for early nodulin gene induction in both nitrogen-fixing and mycorrhizal interactions. So far, three early nodulin genes, *ENOD2*, *ENOD40*, and *ENOD12*, have been shown to be induced by exogenous cytokinin (refs. 43 and 44 and this report). Thus, the expression of these genes serves as a diagnostic marker for a state of altered hormone balance in the root. Because cytokinin induces *ENOD* gene expression, we reasoned that mycorrhizal roots might have higher levels of cytokinin, and indeed some earlier literature had indicated that this was the case (45, 46, 47). We found that the levels of ZR-type cytokinins dramatically increased in mycorrhizal roots 14 days after the start of the experiment; we did not examine roots earlier than this time. It is likely that the level of cytokinin production, by either the fungus or the host, is very low until the infection becomes well established, at which time increased ZR levels are detected. This is coincident with an increase in *ENOD* gene expression (cf. Fig. 2).

Thus, our results suggest that the increase in a ZR-type cytokinin as the mycorrhizal interaction progresses is one of the signals for inducing the early nodulin genes *MsENOD2* and *MsENOD40*. They further suggest that some of the downstream signals, perhaps cytokinin-mediated, are conserved between the *Rhizobium*–legume symbiosis and mycorrhizae. Whether or not the primary signal, a Nod factor-like molecule, is common to both interactions and actually leads to increased cytokinin levels remains to be determined.

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