Medicago truncatula plants overexpressing the early nodulin gene *enod40* exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules

Christian Staehelin*†‡, Celine Charon*, Thomas Boller†, Martin Crespi*, and Ádám Kondorosi*

*Institut des Sciences du Végétal, UPR2355 Centre National de la Recherche Scientifique, F-91198 Gif-sur-Yvette, France; and †Botanisches Institut, Universität Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland

Communicated by R. H. Burris, University of Wisconsin, Madison, WI, September 18, 2001 (received for review December 13, 2000)

The mutualistic symbiosis between flowering plants and arbuscular mycorrhizal fungi is extremely abundant in terrestrial ecosystems. In this symbiosis, obligately biotrophic fungi colonize the root of the host plants, which can benefit from these fungi by enhanced access to mineral nutrients in the soil, especially phosphorus. One of the main goals of research on this symbiosis is to find plant genes that control fungal development in the host plant. In this work, we show that mycorrhizal colonization is regulated by *enod40*, an early nodulin gene known to be involved in the nodule symbiosis of legumes with nitrogen-fixing bacteria. *Medicago truncatula* plants overexpressing *enod40* exhibited stimulated mycorrhizal colonization in comparison with control plants. Overexpression of *enod40* promoted fungal growth in the root cortex and increased the frequency of arbuscule formation. Transgenic lines with suppressed levels of *enod40* transcripts, likely via a cosuppression phenomenon induced by the transgene, exhibited reduced mycorrhizal colonization. Hence, *enod40* might be a plant regulatory gene involved in the control of the mycorrhizal symbiosis.

Most terrestrial flowering plants have the ability to establish symbiotic associations with arbuscular mycorrhizal (AM) fungi. These fungi facilitate the translocation of limiting nutrients, particularly phosphorus, from the soil to the plant root, which in exchange supplies the fungal partner with carbon (1, 2). On contact with the root surface, extraradical hyphae swell and form appressoria. Hyphal penetration of the root occurs via appressoria, and the intraradical hyphae then colonize the root cortex. An appressorium can also be formed without any accompanying infection site. After a few days, the intraradical hyphae penetrate plant cells to form highly branched haustoria, the arbuscules (3). The arbuscules are thought to be the sites where nutrient exchange occurs between the fungus and the host plant. In the late stage of symbiosis, AM fungi develop extraradical hyphae and often vesicles, which are lipid-filled storage structures (1, 2).

One important group of plants that associates with AM fungi is legumes. These plants are especially interesting for research on plant–microbe interactions because they also live in symbiosis with rhizobial bacteria to form nitrogen-fixing root nodules (4, 5). Although these two symbiotic interactions may appear rather different, several similarities have been reported during the colonization process (6, 7). For example, certain flavonoids of the host plant may act as signals promoting the establishment of the fungal as well as the rhizobial symbiosis (8). Moreover, a number of proteins of the host plant are induced in both symbiotic interactions. In *Medicago sativa* for example, the early nodulin gene *enod40* is activated in the nodule primordia (9, 10) and in mycorrhizal roots (11). This gene has numerous short ORFs, and it has been proposed that one of them encodes a small peptide (12). The *enod40* transcript is a structured RNA, which does not associate permanently to polyribosomes (10), and it was suggested that it plays a role in gene function (9). *enod40* is involved in nodulation, as *Medicago truncatula* plants constitutively expressing *enod40* (13) showed accelerated nodule formation, whereas lines cosuppressing the *enod40* transcript exhibited aborted nodule development (14).

Very little is known about plant genes that control mycorrhizal development and arbuscule formation (7), and so far no plant genes have been cloned that regulate the symbiosis with AM fungi. Here, we show that modification of *enod40* expression affected mycorrhizal colonization and arbuscule formation in the root cortex. Hence, *enod40* might be a regulatory gene of the host plant that controls formation of the symbiosis with AM fungi.

Materials and Methods

Biological Material. *M. truncatula* (Gaertner) 108R wild-type plants, the lines Se40(3) and Se40(10) overexpressing a full-length *Mtenod40* cDNA from the 35S promoter and the transgenic lines Se40(2) and Se40(5) with reduced levels of *enod40* transcripts, have been described previously (13, 14). In this study, the following descendants were used: T_3 or T_4 of line Se40(3), T_2 of line Se40(10), T_1 of line Se40(2), and T_2 of line Se40(5). Among the T_2 descendants of line Se40(5), about one-quarter of 10-day-old seedlings exhibited leaf chlorosis. These plants were excluded from the mycorrhizal assays. Wild-type plants regenerated from leaf explants and plants carrying the pG3.3 vector with a cDNA encoding a green fluorescence protein were used for control experiments. The transgenic nature of all tested plant lines was confirmed by Northern blot analysis (ref. 13 and data not shown).

Inoculum of the mycorrhizal fungus *Glomus mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe (fungus collection Botanisches Institut der Universität Basel, Switzerland; an inoculum provided by H. Schüepp, Eidgenössische Versuchsanstalt für Obst-, Wein-, und Gartenbau, Wädenswil, Switzerland) was obtained from Zhi-Ping Xie (15) and further propagated by using *Tagetes tenuifolia* as host plant. *Glomus intraradices* Smith and Schenk (DAOM 197198; Department of Agriculture, Ottawa) was cultivated under *in vitro* conditions with Ri T-DNA transformed carrot (*Daucus carota* L.) roots, kindly provided by Yves Piché (Université Laval, Quebec). Spores were isolated from monoxenic cultures, which were cultivated at 26°C for 8–10 weeks on minimal (M) medium containing 1% gellan gum (Gel-Gro, ICN; ref. 16).

Abbreviation: AM, arbuscular mycorrhizal.

‡To whom reprint requests should be addressed at the present address: Laboratoire de Biologie Moléulaire des Plantes Supérieures, Université de Genève, 1 ch. de l'Impératrice, 1292 Chambésyl Genève, Switzerland. E-mail: Christian.Staehelin@bioveg.unige.ch.

 www.pnas.org/cgi/doi/10.1073/pnas.251491698

Culture System and Growth Conditions. *M. truncatula* seeds were surface sterilized by treatment with diluted commercial bleach (0.35% active chlorine) containing 0.01% SDS at 28°C for 1 h on a rotary shaker at 150 rpm. After thorough washing with sterile H_2O, the seeds were incubated overnight in H_2O at room temperature. They were then washed again with sterile H_2O, and the testa was removed with forceps. Seeds were left to germinate on water agar plates in the dark and transferred to 300-ml magenta bodies after 2 days. The upper vessel was filled with sterile loam and sand in the ratio 1:9 (wt/wt) containing the mycorrhizal inoculum in case of experiments with *G. mosseae*. The phosphorus content of the substrate (after extraction with 0.1 M lactic acid/0.3 M acetic acid/0.1 M ammonium acetate) was 12 mg/kg dry weight, as determined by the molybdate blue ascorbic acid method (17). The lower magenta vessel was filled with autoclaved phosphate-free Fahraeus nutrient solution, pH 6.3, containing 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2 mM K_2SO_4, 1 mM KNO_3, 1 mM Fe(III)-citrate, and the microelements of the Fahraeus medium (18). For experiments with *G. intraradices*, plants were inoculated with a spore suspension (800 spores in 5 ml of H_2O per magenta vessel) 10 days after transfer of the seedlings to the magenta vessels. Plants were cultivated in a greenhouse (experiments with *G. mosseae*; 16 h/day period) or in a growth chamber (experiments with *G. intraradices*; 16-day period, photon flux of 300 μmol·m^{-2}·s^{-1} at 22°C; night period at 18°C). When required, the lower magenta vessels were filled up again with autoclaved phosphate-free Fahraeus nutrient solution.

Staining of Roots and Estimation of Mycorrhizal Colonization. At the time of harvest, the roots were washed with tap water and boiled in 20 ml of 10% (wt/vol) KOH for 5 min, washed with H_2O, and placed in 20 ml of 0.3 M HCl for 2 min. Then, the HCl was poured off, and the roots were incubated for at least 12 h in 10 ml of staining solution containing 0,1% (wt/vol) trypan blue (Aldrich), 24% (vol/vol) glycerin, 31% (vol/vol) lactic acid, and 30% (wt/vol) phenol. For the estimation of mycorrhizal colonization, aliquots of the root material were washed with H_2O, and the degree of mycorrhizal colonization was determined in blind tests using the gridline intersection method with a minimum of 300 line intersections per root sample (19). Data are given as percentages of root length containing blue-stained mycorrhizal structures within the root. This parameter is independent of total root length. External hyphae growing in the vicinity of the roots and sporocarps were not included into the estimation. For microscopical studies, root pieces were washed with a solution containing 24% (vol/vol) glycerin and 31% (vol/vol) lactic acid. The number of mycorrhizal structures (appressoria, arbuscules, vesicles) between two gridlines was determined, and the number of these structures was calculated by using either the total root length (colonized and noncolonized root zones) or root zones colonized by AM fungi as a basis. The size of a colonization zone in young roots was determined by measuring the root length carrying mycorrhizal structures including intraradical hyphae.

Experimental Design and Statistical Analysis. The aim of this work was to test whether transcript levels of *enod40* influence mycorrhizal colonization. To test this, seven different experiments were executed (see *Results* for details). In each experiment, different plant lines with altered levels of *enod40* transcripts were compared with wild-type plants. Per plant line (including the wild-type), five (or more) magenta vessels were prepared, which thus gave at least five replicates per treatment. Eleven (or more) replicates per treatment were used for experiment 6. Where plants were grown together in magenta vessels, plant material from each vessel was combined for data collection, and pooled means from each vessel were used for statistical analysis (S-PLUS 4.5 program from MathSoft, Bagshot, Surrey, U.K.). Because of nonnormality within treatment and to variance inequality among treatments in some experiments, data were analyzed by the nonparametric Kruskal–Wallis rank sum test, which is also suitable for unequal replication (20). The Kruskal–Wallis statistics is approximately distributed as a $\chi^2_{(a-1)}$, with a being the number of treatments that were compared. Pairwise and multiple comparisons among plant lines were consequently executed to test whether treatments varied from each other by the Kruskal–Wallis test. A probability of $P < 0.05$ was considered as representing a significant difference in this study. All data given are means ± SE.

Results

Transgenic *M. truncatula* Plants Overexpressing *enod40* Exhibit Increased Colonization by *G. mosseae*. In a first series of experiments, root colonization of *M. truncatula* plants by the fungus *G. mosseae* was examined. The degree of mycorrhizal colonization was significantly higher in roots of the *enod40*-overexpressing line Se40(3) in comparison with wild-type plants ($n = 10$, $\chi^2_{[1]} = 6.81$, $P = 0.009$). Plants regenerated from leaf explants and transgenic plants carrying the pG3.3 vector containing a 35S promoter–*GFP* construct were used as additional controls. Mycorrhizal colonization in these plants was as low as in the wild-type plants, indicating that increased colonization was correlated with *enod40* overexpression and was independent of the transformation and regeneration procedure (experiment 1; three plants per magenta vessel; Fig. 1*a*).

To test fungal colonization during the development of the root system in wild-type and *enod40*-overexpressing plants, mycorrhizal plants were harvested at various time points p.i. (experiment 2; three plants per magenta vessel). The root system of *M. truncatula* plants was progressively colonized by *G. mosseae* (Fig. 1*b*). Compared with wild-type plants, the *enod40*-overexpressing line Se40(3) exhibited an accelerated mycorrhizal colonization, and a difference was already seen 12 days p.i. In this early stage of symbiosis, fungal colonization of line Se40(3) was nearly 2-fold higher in comparison with wild-type plants. At later stages, the degree of fungal colonization of line Se40(3) was about 10% higher than that of wild-type plants. This increase corresponded to an acceleration of mycorrhizal colonization of about 1 week. The difference in mycorrhizal colonization was statistically different between the two lines at each of the first four time points (12 days p.i., $n = 14$, $\chi^2_{[1]} = 9.24$, $P = 0.002$; 18 days p.i., $n = 10$, $\chi^2_{[1]} = 4.81$, $P = 0.028$; 28 days p.i., $n = 10$, $\chi^2_{[1]} = 3.94$, $P = 0.047$; 36 days p.i., $n = 10$, $\chi^2_{[1]} = 5.77$, $P = 0.016$) (Fig. 1*b*).

The quantity of fungal inoculum is a parameter that influences mycorrhizal root colonization. To test the effect of *enod40* overexpression on *M. truncatula* plants with a low degree of mycorrhizal colonization, plants were infected with another inoculum of *G. mosseae*, which was added to the growth medium at 50-fold lower amounts compared with the other experiments. As expected, fungal colonization was delayed, and the rate of root colonization after 35 days was still low. The difference between the degree of fungal colonization of the line Se40(3) and the wild-type plants was also seen under these conditions (experiment 3; four plants per magenta vessel; $n = 10$, $\chi^2_{[1]} = 6.32$, $P = 0.012$) (Fig. 1*c*). A similar experiment was performed with line Se40(10), another *enod40*-overexpressing *M. truncatula* line, and the root material was harvested 49 days p.i. (experiment 4; four plants per magenta vessel). Similarly to line Se40(3), roots from line Se40(10) exhibited enhanced mycorrhizal colonization in comparison with the wild-type plants ($n = 10$, $\chi^2_{[1]} = 5.77$, $P = 0.016$) (Fig. 1*d*). These data indicate that *enod40* overexpression promotes

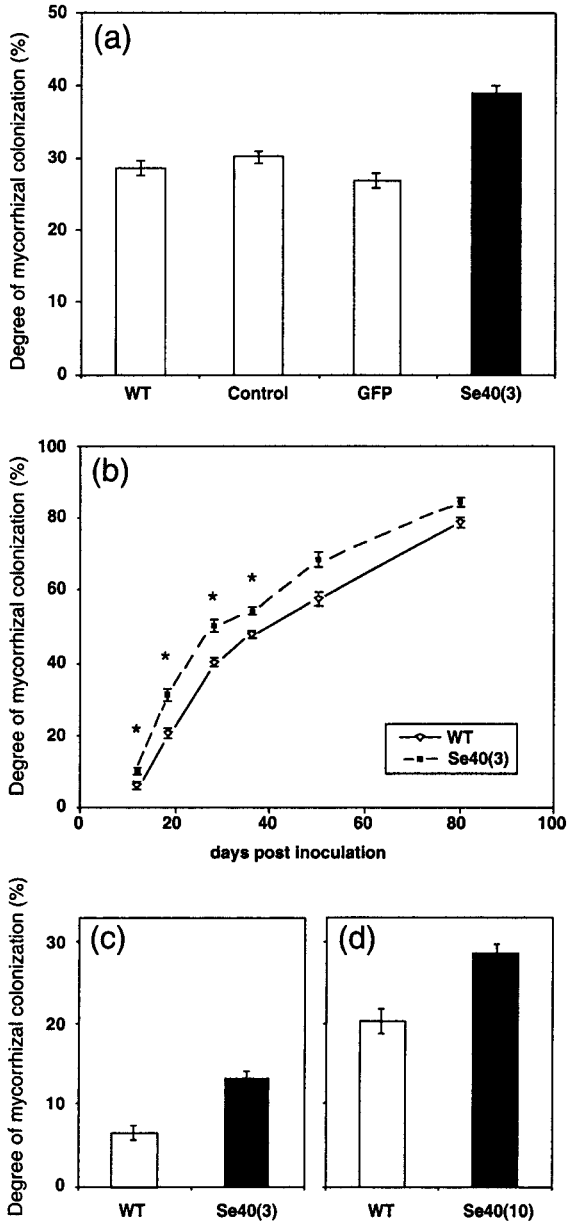

Fig. 1. Transgenic *M. truncatula* plants overexpressing *Mtenod40* exhibit enhanced colonization by *G. mosseae*. (*a*) Degree of mycorrhizal colonization (length of roots containing mycorrhizal structures in percentage of total root length) of *M. truncatula* wild-type (WT) plants, WT plants regenerated from leaf explants (Control), plants expressing a 35S promoter-*GFP* fusion (GFP), and line Se40(3) overexpressing the *Mtenod40* gene harvested 21 days p.i. (experiment 1; $n = 20$, $\chi^2_{[3]} = 12.58$, $P = 0.006$). (*b*) Time course of the mycorrhizal colonization of WT and Se40(3) plants (experiment 2). The degree of mycorrhizal colonization was estimated at different time points after inoculation. Differences (indicated by asterisks) were statistically significant ($P < 0.05$) up to 38 days. (*c* and *d*) Mycorrhizal colonization by *G. mosseae* using low amounts of inoculum. The difference between WT and two different *enod40*-overexpressing lines was significant ($P < 0.05$) in both experiments. (*c*) Degree of mycorrhizal colonization in roots of WT plants and line Se40(3) harvested 35 days p.i. (experiment 3). (*d*) Degree of mycorrhizal colonization in roots of WT plants and line Se40(10) harvested 49 days p.i. (experiment 4).

mycorrhizal colonization in two independent *enod40*-overexpressing *M. truncatula* lines.

Overexpression of *enod40* Stimulates Fungal Growth *in Planta*. To determine whether *enod40* overexpression affects the initial stages of mycorrhizal colonization, comparative microscopical studies on *enod40*-overexpressing and wild-type plants from experiment 2 were performed. As shown in Fig. 1*b*, mycorrhizal colonization by *G. mosseae* was observed as early as 12 days p.i. At this stage, total root length of both plants was about 30 cm, and the first small fungal colonization zones were seen. The average number of such separated colonization zones per plant was similar in wild-type and in the *enod40*-overexpressing line Se40(3) (Table 1). The average size of a colonization zone, however, as determined by its length, was significantly increased in the Se40(3) line ($n = 14$, $\chi^2_{[1]} = 5.40$, $P = 0.020$). Consequently, the total root length colonized by *G. mosseae* was higher in the Se40(3) line compared with the wild-type plants ($n = 14$, $\chi^2_{[1]} = 4.27$, $P = 0.039$). The number of appressoria (per cm total root length) was similar for wild-type and *enod40*-overexpressing plants. The number of appressoria per mm colonized root zone, however, was lower in the *enod40*-overexpressing line Se40(3) in comparison with the wild-type ($n = 14$, $\chi^2_{[1]} = 4.54$, $P = 0.033$). Taken the data of Table 1 together, they show that overexpression of *enod40* promotes fungal growth *in planta*.

Table 1. Mycorrhizal colonization zones and frequency of appressoria formation in roots of *M. truncatula* wild-type plants and line Se40(3) overexpressing the *Mtenod40* gene

	Wild type	Se40(3)
Number of independent colonization zones (per plant)	4.5 ± 0.7 a	4.4 ± 0.3 a
Average length of a colonization zone, mm	4.1 ± 0.6 a	6.3 ± 0.7 b
Total length, in mm, of the colonization zones (per plant)	18.2 ± 2.9 a	28.0 ± 2.0 b
Number of appressoria per cm (total root length)	0.36 ± 0.06 a	0.38 ± 0.05 a
Number of appressoria per mm (in colonized root zones)	0.60 ± 0.07 a	0.37 ± 0.06 b

Plants harvested 12 days p.i. with *G. mosseae* were analyzed (experiment 2). Values in lines followed by different letters are significantly different ($P < 0.05$).

Overexpression of *enod40* Promotes Development of Arbuscules. To investigate whether *enod40* overexpression affects formation of arbuscules, the number of arbuscules was counted on roots harvested 12 and 18 days p.i. (experiment 2). Compared with wild-type plants, *enod40*-overexpressing plants exhibited a significantly higher number of arbuscules, when calculated per mm total root length (12 days p.i., $n = 14$, $\chi^2_{[1]} = 9.60$, $P = 0.002$; 18 days p.i., $n = 10$, $\chi^2_{[1]} = 6.32$, $P = 0.012$) (Fig. 2*a*). We also determined the number of arbuscules per mm colonized root (Fig. 2*b*). In roots harvested 12 days p.i., most arbuscules were formed in the inner cortical cell layers that were adjacent to the endodermis. The frequency of arbuscule formation in colonized root zones of *enod40*-overexpressing plants was significantly higher in comparison with the wild-type plants ($n = 14$, $\chi^2_{[1]} = 6.01$, $P = 0.014$), indicating that increased formation of arbuscules in *enod40*-overexpressing plants was not simply because of an increase in the overall length of root colonized by *G. mosseae*. For roots harvested 18 days p.i., this difference was no longer significant (Fig. 2*b*). Taken together, these data show that *enod40* overexpression resulted in enhanced formation of arbuscules in young roots.

We also compared the number of vesicles in *enod40*-overexpressing and wild-type roots. Only few vesicles were seen in roots harvested 12 days p.i. Because of the increased fungal colonization rate in *enod40*-overexpressing plants, the number of vesicles per mm total root length was higher in comparison with

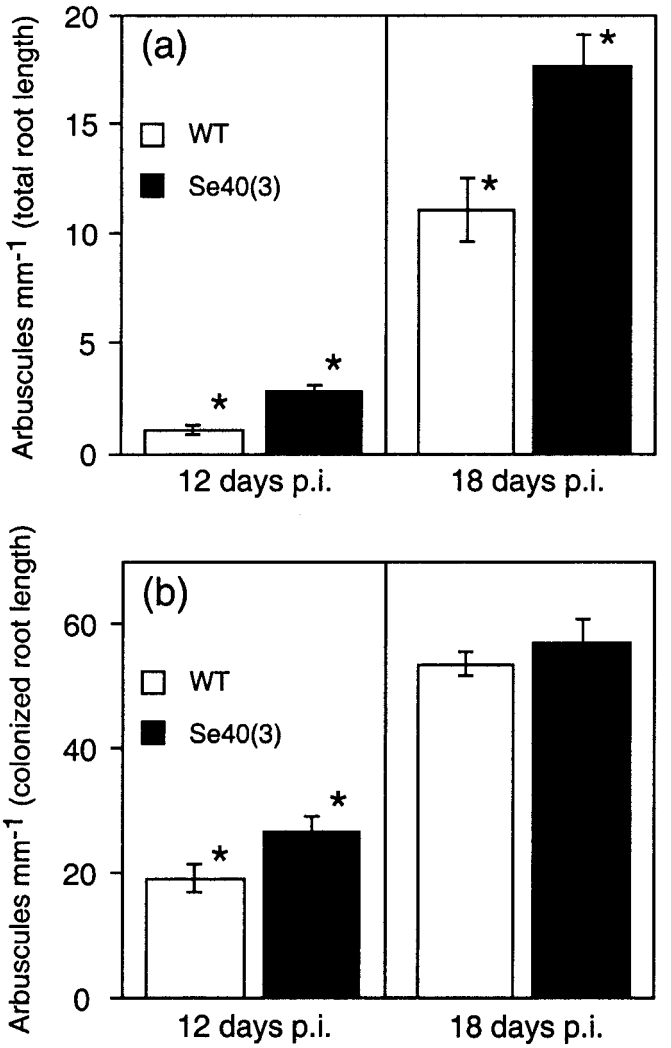

Fig. 2. Number of arbuscules in wild-type (WT) plants and line Se40(3) overexpressing *enod40*. Plants were inoculated with *G. mosseae* and harvested 12 and 18 days later (experiment 2). Asterisks indicate statistically significant differences ($P < 0.05$) between WT and line Se40(3). (*a*) Number of arbuscules (per mm root length) on the basis of the total root length (colonized and noncolonized root zones). (*b*) Number of arbuscules (per mm root length) in root zones colonized by *G. mosseae*.

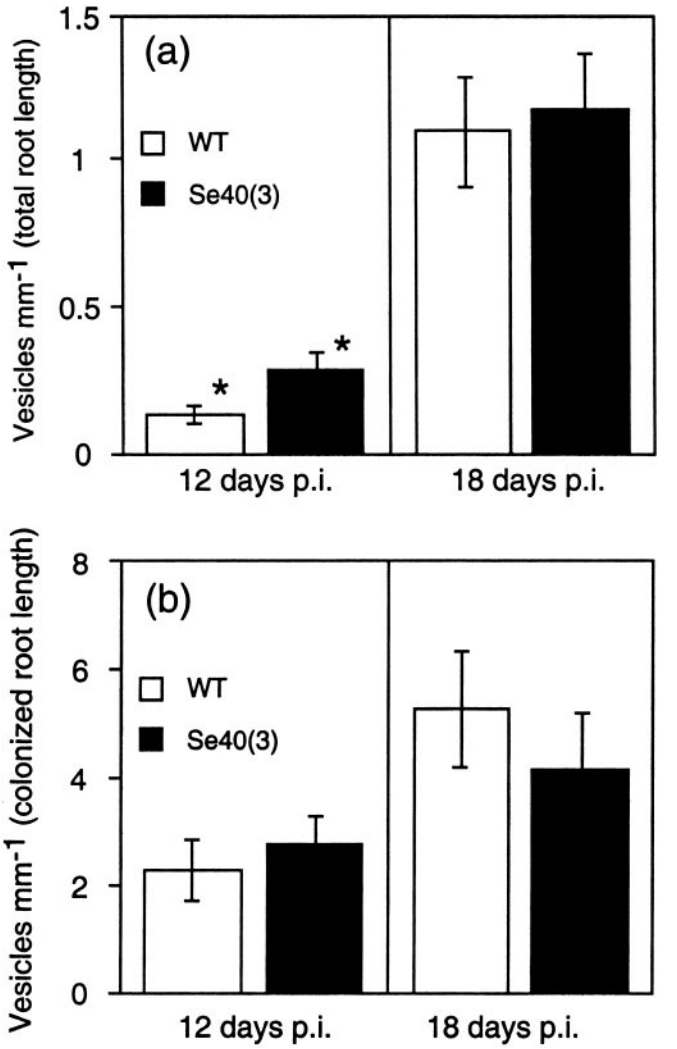

Fig. 3. Number of vesicles in wild-type (WT) plants and line Se40(3) overexpressing *enod40*. Plants were harvested 12 and 18 days after inoculation with *G. mosseae* (experiment 2). (*a*) Number of vesicles (per mm root length) on the basis of the total root length (colonized and noncolonized root zones). Asterisks indicate statistically significant differences ($P < 0.05$) between WT and line Se40(3) for root material harvested 12 days p.i. (*b*) Number of vesicles (per mm root length) in root zones colonized by *G. mosseae*.

the wild-type plants ($n = 14$, $\chi^2_{[1]} = 4.82$, $P = 0.028$). In roots harvested 18 days p.i., no significant differences were seen (Fig. 3*a*). In contrast to the formation of arbuscules (Fig. 2*b*), *enod40* overexpression did not affect the frequency of vesicle formation in colonized root zones (Fig. 3*b*).

***M. truncatula* Plants with Suppressed Levels of *enod40* Transcripts Exhibit Reduced Colonization by *G. intraradices*.** We also tested mycorrhizal colonization of *M. truncatula* by another fungus. Plants were inoculated with *G. intraradices* spores and cultivated in a growth chamber. In this system, spore germination was required and fungal colonization of young roots was lower than in the previous experiments with *G. mosseae*. Similarly to the association with *G. mosseae*, mycorrhizal colonization by *G. intraradices* was stimulated in the *enod40*-overexpressing line Se40(3) ($n = 10$, $\chi^2_{[1]} = 3.93$, $P = 0.047$) or in the line Se40(10) ($n = 11$, $\chi^2_{[1]} = 4.80$, $P = 0.029$) when compared with wild-type plants. Transgenic control plants carrying the pG3.3 vector with a 35S promoter-*GFP* did not exhibit enhanced fungal colonization (experiment 5; three plants per magenta vessel; Fig. 4*a*). In another experiment, we tested the transgenic *M. truncatula* lines Se40(2) and Se40(5), which show reduced *enod40* transcript accumulation (13). This reduction in transcript levels is probably as a result of cosuppression induced by the transgene (21). Compared with wild-type plants, mycorrhizal colonization of line Se40(2) ($n = 36$, $\chi^2_{[1]} = 4.85$, $P = 0.027$) and line Se40(5) ($n = 28$, $\chi^2_{[1]} = 6.34$, $P = 0.012$) was significantly lower, whereas the *enod40*-overexpressing Se40(3) line exhibited stimulated fungal colonization ($n = 32$, $\chi^2_{[1]} = 7.60$, $P = 0.006$) (experiment 6; one plant per magenta vessel) (Fig. 4*b*). These results indicate that the amount of *enod40* transcript accumulation alters the degree of mycorrhizal colonization.

Symbiotic Efficiency. Mycorrhizal colonization of legumes can promote plant growth responses, indicating a benefit of the host plant from the symbiosis (22, 23). To examine the mycorrhizal responsiveness of *M. truncatula* under our growth conditions, noninoculated plants and mycorrhizal plants colonized by *G.*

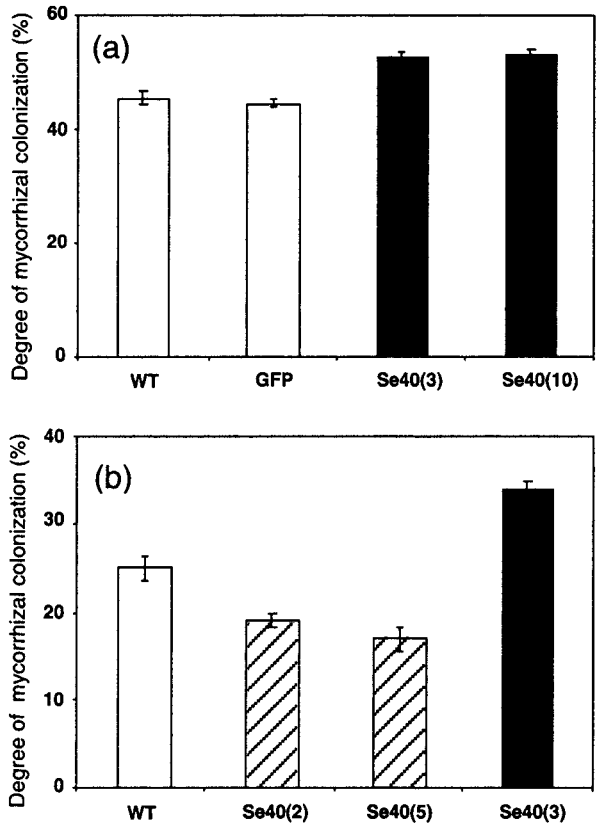

Fig. 4. Mycorrhizal colonization by *G. intraradices*. (*a*) Degree of mycorrhizal colonization of *M. truncatula* wild-type (WT) plants, plants expressing a 35S promoter-*GFP* fusion (GFP) and overexpressing the *Mtenod40* gene [lines Se40(3) and Se40(10)] harvested 56 days p.i. (experiment 5; $n = 22$, $\chi^2_{[3]} = 12.39$, $P = 0.006$). (*b*) Degree of mycorrhizal colonization of *M. truncatula* wild-type (WT) plants, two lines with a cosuppression phenomenon [lines Se40(2) and Se40(5)] and line Se40(3) overexpressing *enod40* harvested 49 days p.i. (experiment 6; $n = 62$, $\chi^2_{[3]} = 27.17$, $P < 0.001$).

mosseae were harvested 42 days p.i., and their dry weight was determined (experiment 7). Independently of the inoculation with the fungus, wild-type plants and the *enod40*-overexpressing Se40(3) line showed similar values for total dry weight per plant [noninoculated wild type, 32.4 ± 5.1 mg; inoculated wild type, 32.3 ± 4.0 mg; noninoculated line Se40(3), 36.7 ± 7.6 mg; inoculated line Se40(3), 41.1 ± 8.7 mg]. Statistical analysis revealed no significant differences, indicating that the amount of produced plant biomass was independent of mycorrhizal colonization under our test conditions. No significant differences were also seen when dry weight values for roots and shoots were compared (data not shown). As expected, mycorrhizal plants exhibited a ≈2-fold and significant increase in their phosphorus content in comparison with noninoculated control plants, but no significant difference was found between line Se40(3) and wild-type plants (data not shown). These data indicate that *enod40* transcript levels in *M. truncatula* plants did not affect symbiotic efficiency, even though alteration of *enod40* expression significantly influenced the degree of root colonization by AM fungi.

Discussion

This paper shows that alteration in the transcript level of the *enod40* gene has an effect on the growth of mycorrhizal fungi in the root and on the development of cells containing arbuscules. Effects of *enod40* transcript levels on nodule formation of *M. truncatula* have been reported (14). These findings indicate that *enod40* may have a general role in mutualistic endosymbioses. This is in line with the genetic evidence, obtained from symbiosis-defective mutants, that certain *sym* genes of the host plant control both rhizobial and mycorrhizal symbioses (6, 7).

In mycorrhizal *M. sativa* roots, *in situ* hybridization studies showed accumulation of *enod40* transcripts in the pericycle, in the epidermis, as well as in young arbuscules (11). In general, *enod40* transcripts were found in cells that transport carbohydrates to strong sinks or become those sinks, such as the nodule and lateral root primordium or the peripheral cells of vascular bundles (9, 10, 24, 25). Indeed, in developing rice leaves, *enod40* expression changed during the source/sink transition (26). One possibility is that *enod40* transcripts in mycorrhizal roots are involved in the control of carbohydrate supply or partitioning into specific cells. Fungal growth in the root and formation of arbuscules are two closely linked processes that depend on carbon availability. Increased carbohydrate supply to the fungus could be a reason why *M. truncatula* plants overexpressing *enod40* exhibited accelerated mycorrhizal colonization. Alternatively, *enod40* transcripts might stimulate fungal growth in the root cortex by affecting targets that interfere with plant hormone action and unknown cell-to-cell communication processes between vascular and cortical tissues. It is worth noting in this context that the amount of *enod40* transcripts in *M. sativa* can be enhanced by treatment of roots with cytokinins and rhizobial Nod factors as well (9, 11, 27). It is possible therefore that *enod40* in mycorrhizal roots is activated via hormonal imbalances. We suggest that the reported promoting effects of purified Nod factors (15) and auxin transport inhibitors (28) on mycorrhizal colonization are connected to activation and action of *enod40*.

During rhizobial infection, a transient increase in the root length of *enod40*-overexpressing plants was observed (14). In the experiments of this study, we did not find any significant difference in root length of plants colonized by AM fungi. It should be considered that large root systems developed under our culture conditions for mycorrhizae. Initial AM colonization 12 days p.i. was detected on 30-cm-long root systems, where slight differences in root length could not be easily measured. Under our test conditions, *M. truncatula* plants also did not show increased biomass acquisition compared with noncolonized plants, indicating a neutral mycorrhizal dependency of this species. Future studies on plant species with high mycorrhizal dependency are required to find a possible benefit from enhanced *enod40* transcript levels on plant growth and crop yield.

Future studies will also focus on the question whether *enod40 per se* is an essential element for mycorrhizal colonization. An approach would be to identify *enod40* genes in various host and nonhost plants of AM fungi. Interestingly, *enod40* genes are not restricted to legumes. Homologs of *enod40* are known from tobacco (12) and rice (26), host plants of AM fungi, but were not found in the genome of the nonhost *Arabidopsis thaliana*. A search for *enod40* homologs from various nonlegumes could result in a correlation between the finding of *enod40* sequences and host plants. Further efforts are also required to study mycorrhizal colonization in *enod40*-transformed nonlegumes, including nonhost plants. Our recent observations indicate, however, that expression of *Mtenod40* in the nonhost *A. thaliana* does not promote growth of *G. intraradices* in the rhizosphere.

Taken together, this study shows that transcript levels of a plant gene affect root colonization by AM fungi and formation of arbuscules. This increases our knowledge in understanding the molecular mechanisms and how plants regulate the symbiotic interaction with mycorrhizal fungi.

We express our gratitude to Yves Piché (Centre de Recherche en Biologie Forestière, Université Laval, Quebec) for the monoxenic *G. intraradices*/carrot culture. We thank Eva Kondorosi (Centre National de la Recherche

Scientifique, Institut des Sciences du Végétal, Gif-sur-Yvette, France), Andres Wiemken (Botanisches Institut der Universität Basel, Switzerland), and William J. Broughton (Université de Genève, Switzerland) for helpful discussions. We are indebted to Yamama Naciri-Graven (Conservatoire et Jardin Botaniques de Genève, Switzerland) for help in statistical analysis. We are grateful to Ann M. Hirsch (University of California, Los Angeles) and Marcel G. A. van der Heijden (Free Universiteit, Amsterdam) for helpful comments on the manuscript. This work was supported in part by a grant from the Novartis Foundation Basel (to C.S.), the Max-Planck-Gesellschaft research award (to A.K.), and the Ministère Français de l'Enseignement Supérieur et de la Recherche (to C.C.).

1. Harrison, M. J. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 361–389.
2. Smith, S. E. & Read, D. J. (1997) *Mycorrhizal Symbioses* (Academic, London), 2nd Ed.
3. Blee, K. A. & Anderson, A. J. (1998) *Plant J.* **16,** 523–530.
4. Schultze, M. & Kondorosi, A. (1998) *Annu. Rev. Genet.* **32,** 33–57.
5. Perret, X., Staehelin, C. & Broughton, W. J. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 180–201.
6. Hirsch, A. M. & Kapulnik, Y. (1998) *Fungal Genet. Biol.* **23,** 205–212.
7. Marsh, S. F. & Schultze, M. (2001) *New Phytol.* **150,** 525–532.
8. Vierheilig, H., Bago, B., Albrecht, C., Poulin, M. J. & Piché, Y. (1998) in *Flavonoids in the Living System*, eds. Manthey, J. & Buslig, B. (Plenum, New York), pp. 9–33.
9. Crespi, M. D., Jurkevitch, E., Poiret, M., D'Aubenton-Carafa, Y., Petrovics, G., Kondorosi, E. & Kondorosi, A. (1994) *EMBO J.* **13,** 5099–5112.
10. Asad, S., Fang, Y., Wycoff, K. L. & Hirsch, A. M. (1994) *Protoplasma* **183,** 10–23.
11. Van Rhijn, P., Fang, Y., Galili, S., Shaul, O., Atzmon, N., Wininger, S., Eshed, Y., Lum, M., Li, Y., To, V., *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5467–5472.
12. Van de Sande, K., Pawlowski, K., Czaja, I., Wieneke, U., Schell, J., Schmidt, J., Walden, R., Matvienko, M., Wellink, J., Van Kammen, A., *et al.* (1996) *Science* **273,** 370–373.
13. Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A. & Crespi, M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8901–8906.
14. Charon, C., Sousa, C., Crespi, M. & Kondorosi, A. (1999) *Plant Cell* **11,** 1953–1965.
15. Xie, Z.-P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W. J., Vögeli-Lange, R. & Boller, T. (1995) *Plant Physiol.* **108,** 1519–1525.
16. Bécard, G. & Fortin, J. A. (1988) *New Phytol.* **108,** 211–218.
17. Watanabe, F. S. & Olsen, S. R. (1965) *Soil Sci. Soc. Proc.* **29,** 677–678.
18. Fahraeus, G. (1957) *J. Gen. Microbiol.* **16,** 374–381.
19. Giovannetti, M. & Mosse, B. (1980) *New Phytol.* **84,** 489–500.
20. Sokal, R. R. & Rohlf, F. J. (1995) *Biometry: The Principles and Practice of Statistics in Biological Research* (Freeman, New York), 3rd Ed.
21. Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Mourrain, P., Palauqui, J.-C. & Vernhettes, S. (1998) *Plant J.* **16,** 651–659.
22. van der Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A. & Sanders, I. R. (1998) *Nature (London)* **396,** 69–72.
23. Wilson, G. W. T. & Hartnett, D. C. (1998) *Am. J. Bot.* **85,** 1732–1738.
24. Yang, W. C., Katinakis, P., Hendriks, P., Smolders, A., de Vries, F., Spee, J., van Kammen, A. & Bisseling T. (1993) *Plant J.* **3,** 573–585.
25. Minami, E., Kouchi, H., Cohn, J. R., Ogawa, T. & Stacey, G. (1996) *Plant J.* **10,** 23–32.
26. Kouchi, H., Takane, K.-I., So, R. B., Ladha, J. K. & Reddy, P. M. (1999) *Plant J.* **18,** 121–129.
27. Fang, Y. W. & Hirsch, A. M. (1998) *Plant Physiol.* **116,** 53–68.
28. Xie, Z.-P., Müller, J., Wiemken, A., Broughton, W. J. & Boller, T. (1998) *New Phytol.* **139,** 361–366.