Suppression of Endochitinase, β-1,3-Endoglucanase, and Chalcone Isomerase Expression in Bean Vesicular-Arbuscular Mycorrhizal Roots Under Different Soil Phosphate Conditions

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Vesicular-arbuscular (VA) mycorrhizal fungi colonize the root cortex successfully under low soil phosphate (P) conditions, whereas infection is delayed or aborted under high P conditions. We evaluated the possibility of an increased expression of endochitinase, β-1,3-endoglucanase, and phytoalexin pathway enzymes that might contribute to inhibition of intraradical fungal growth under high P concentrations. Under low and high P concentrations, bean mycorrhizal roots showed an early induction of endochitinase activity followed by suppression relative to the noninfected controls. Under low P, maximal suppression occurred during the period of maximal fungal growth rate. Suppression of endochitinase activity was attenuated under high P concentration (up to 1.9-fold) compared with the suppression under low P (up to 3.7-fold). Suppression under both conditions was accompanied by differential reductions in the levels of mRNAs encoding two endochitinase isoforms. Suppression of β-1,3-endoglucanase activity was also observed at certain stages of the mycorrhiza development under both P concentrations. The levels of two β-1,3-endoglucanase mRNAs encoding distinct isoforms were differentially suppressed (up to 13-fold) in response to mycorrhiza formation under both P levels. In addition, the chalcone isomerase mRNA level was suppressed (up to 2.4-fold) in mycorrhizal roots under both P concentrations. Our data suggest that there is a general suppression of the plant defense response during VA mycorrhiza development. Possible mechanisms of regulation and functions of endochitinase and β-1,3-endoglucanase in VA mycorrhiza are discussed.

Additional keywords: chalcone synthase, phenylalanine ammonia-lyase.

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The vesicular-arbuscular (VA) mycorrhizal fungi, which are members of the class Zygomycetes, order Glomales, form symbiotic associations with roots and enhance plant growth primarily by increasing the absorption of mineral nutrients. The colonization process is characterized by intercellular and intracellular hyphal growth in the cortical tissue, with differentiation of terminal intracellular hypha into ephemeral, haustoriumlike structures called arbuscules. Despite extensive fungal colonization, there is no generalized hypersensitive response in VA mycorrhizal roots (Gianinazzi 1991) similar to the symbiosis between members of the Rhizobiaceae and legumes. Previous studies have shown that chitinase and peroxidase activities are higher in leek mycorrhizal roots during the early stages of infection and are later suppressed to levels below the noninfected control (Spanu and Bonfante-Fasolo 1988; Spanu *et al.* 1989). In addition, it has been shown that phytoalexins accumulate to a minor extent in soybean mycorrhizal roots (Morandi *et al.* 1984; Wyss *et al.* 1991).

One factor that has been shown to limit fungal colonization and sporulation is high phosphate (P) concentration (Sanders 1975). High concentrations of P inhibit intraradical fungal growth (Lambais and Cardoso 1988), possibly through P-mediated physiological alterations of the roots (Koide and Li 1990). Induction of plant defense genes in the roots may be one factor in reducing colonization in soils containing high P concentrations. It has been shown that P applied to cucumber leaves induces the expression of chitinase and peroxidase both locally and systemically (Irving and Kuć 1990).

The aim of this study was to test the hypothesis that reduced root colonization in soil containing a high P concentration is due to an increased expression of defense-related genes. As a model system, we used bean infected with *Glomus intraradices* grown at two P concentrations and studied the expression of genes encoding endochitinases, β-1,3-endoglucanases, and phenylpropanoid pathway enzymes necessary for phytoalexin synthesis. Increased expression of these mRNAs at a high P concentration would be predicted to occur as either an induction above basal levels or as a smaller suppression of expression in the presence of mycorrhizal fungi.

We found that endochitinase and β-1,3-endoglucanase activities were suppressed at certain stages during VA

mycorrhiza development under both low and high P concentrations. The suppression of enzyme activities under both P conditions was accompanied by suppression in the levels of mRNAs encoding specific isoforms of these enzymes. Our data suggest that there is a general suppression of plant defense mechanisms during VA mycorrhiza development and that the suppression of endochitinase activity is attenuated under high P concentration. In addition, we found that the levels of the mRNA encoding chalcone isomerase, an enzyme in the phenylpropanoid pathway, were suppressed in bean mycorrhizal roots under both P concentrations. Under high P concentrations, the relatively higher levels of endochitinase activity might contribute to the restricted intraradical fungal growth but would not likely be sufficient to explain the effect of P on the root colonization process.

RESULTS

Bean growth and infection parameters.

In order to make comparisons of enzyme activity and mRNA accumulation between mycorrhizal and noninfected plants, it was important to verify that the two sets of plants had approximately comparable growth characteristics (Table 1). At the low P concentration, mycorrhizal plants produced approximately 16% less root biomass than noninfected plants at 8 and 10 weeks after planting (WAP). Root lengths of both sets of plants were very similar at all harvests. At the high P concentration, mycorrhizal plants had approximately 22% less root biomass than noninfected plants at 6, 8, and 10 WAP. In addition, root lengths were comparable, except at 10 WAP, when mycorrhizal plants showed a 20% decrease in root length. Thus, root and shoot growth were similar overall for mycorrhizal and noninfected plants.

In plants grown at the low P concentration, colonization by *G. intraradices* was low at 4 WAP, then increased rapidly and reached a plateau at 8 WAP (Table 1). At the high P concentration, the colonization rate was reduced by 50% at 4 WAP and by approximately 80% at later weeks in comparison with those grown at low P.

Chitinase activities.

At the low soil P concentration, noninfected bean roots showed increases in endochitinase specific activity with plant development (Fig. 1A). Mycorrhizal roots contained significantly more endochitinase activity than noninfected roots at 4 WAP and less during all of the other weeks (t test, $P = 0.05$). Endochitinase activity in mycorrhizal roots was approximately 1.6-fold higher at 4 WAP and 1.8-, 3.7-, and 1.9-fold lower at 6, 8, and 10 WAP, respectively, than noninfected controls.

Figure 1B shows that exochitinase activity in noninfected roots also increased with developmental age and constituted between 3 and 16% of the total chitinase activity (Fig. 1B and C). However, in contrast to endochitinase, mycorrhizal roots exhibited 2.1-fold higher exochitinase activity at 6 and 8 WAP than noninfected roots (t test, $P = 0.05$). The pattern of total chitinase activity in mycorrhizal and noninfected roots was similar to the pattern observed for endochitinase (Fig. 1C).

At the high soil P concentration, endochitinase activity was also developmentally regulated, with maximum activity at 8 WAP. As shown for plants grown under low P, endochitinase specific activity was higher in mycorrhizal roots than in noninfected controls at 4 WAP and lower at the other weeks. Endochitinase specific activity was 1.6-fold higher at 4 WAP and 1.5- to 1.9-fold lower at subsequent weeks than noninfected roots. Noninfected roots at the high P concentration showed higher levels

Table 1. Plant growth and infection parameters of mycorrhizal and noninfected bean plants grown at low or high phosphate concentration

Phosphate level WAP[a]	Treatment[b]	Root fresh weight[c] (g)	Shoot dry weight (g)	Root length (cm)	Percent of total root length infected
Low					
4	Noninfected	3.36 ± 0.11[c]	1.10 ± 0.06	263 ± 21	0
	Mycorrhizal	3.62 ± 0.20	0.98 ± 0.05	241 ± 21	5.58 ± 1.23
6	Noninfected	4.46 ± 0.24	1.65 ± 0.09	303 ± 21	0
	Mycorrhizal	4.66 ± 0.28	1.46 ± 0.09	270 ± 20	33.02 ± 3.89
8	Noninfected	6.31 ± 0.30	1.96 ± 0.13	265 ± 14	0
	Mycorrhizal	5.35 ± 0.43	2.49 ± 0.08	292 ± 14	69.21 ± 2.49
10	Noninfected	6.97 ± 0.40	2.20 ± 0.20	257 ± 15	0
	Mycorrhizal	5.72 ± 0.37	2.49 ± 0.08	292 ± 12	71.80 ± 2.42
High					
4	Noninfected	3.37 ± 0.07	1.04 ± 0.05	265 ± 18	0
	Mycorrhizal	3.16 ± 0.19	1.11 ± 0.04	231 ± 12	2.80 ± 0.72
6	Noninfected	4.99 ± 0.37	1.75 ± 0.09	298 ± 15	0
	Mycorrhizal	3.92 ± 0.19	1.79 ± 0.07	255 ± 16	5.97 ± 0.49
8	Noninfected	5.54 ± 0.19	3.34 ± 0.06	265 ± 12	0
	Mycorrhizal	4.28 ± 0.17	3.19 ± 0.13	264 ± 16	12.82 ± 1.43
10	Noninfected	6.79 ± 0.38	4.21 ± 0.16	342 ± 16	0
	Mycorrhizal	5.27 ± 0.44	3.69 ± 0.12	273 ± 15	17.81 ± 1.67

[a] Weeks after planting.
[b] Mycorrhizal plants were infected with *Glomus intraradices* strain 25.
[c] Average of 10 plants ± standard deviation of the mean.

of endochitinase activity than at the low P concentration at 4 and 8 WAP (t test, $P = 0.05$), whereas in mycorrhizal roots significant increases were observed at 4, 6, and 8 WAP (t test, $P = 0.05$). At the high P concentration, exochitinase activity was 2.4-fold lower in mycorrhizal roots than in noninfected roots at 8 WAP (Fig. 1D). Figure 1F presents the comparison of total chitinase activities.

To determine whether the observed suppression in endochitinase activity was due to the presence of an inhibitor in the crude enzyme extracts from mycorrhizal roots, we performed a series of mixing experiments. Equal amounts of crude enzyme extract from mycorrhizal and noninfected roots at different times were mixed, and chitinase activities were determined. The expected endochitinase specific activities did not differ from the measured activities (data not shown), indicating that the suppression in mycorrhizal roots under both P conditions was not due to the presence of inhibitors.

Chitinase mRNAs.

The steady-state levels of specific basic and acidic endochitinase mRNAs were evaluated by Northern blot analysis of poly(A)$^+$ RNA isolated from the same samples used for the enzymatic assays. A full-length clone, designated CHT476, isolated from an elicitor-treated bean cell culture cDNA library (Sheng and Mehdy, unpublished) was identical in the coding region nucleotide sequence to a previously reported bean CH5B cDNA clone encoding a basic endochitinase (Broglie *et al.* 1986). CH5B-hybridizable mRNA has been shown to be induced by ethylene and fungal elicitor in leaves (Broglie *et al.* 1986; Roby *et al.* 1991) and by pathogen attack and wounding in hypocotyls (Hedrick *et al.* 1988). Figure 2A shows that CHT476-hybridizable mRNA accumulated to a maximum at 8 WAP in the roots of noninfected plants grown in low P soil. Mycorrhizal roots exhibited 1.5- and 2.2-fold less RNA at 4 and 6 WAP, respectively, than the noninfected control and little differences at later weeks.

At the high soil P concentration, noninfected and mycorrhizal roots showed comparable, increasing levels of CHT476 transcripts throughout the time course (Fig. 2A). The levels of CHT476 transcripts in noninfected high P roots were approximately two- and sixfold higher at 4 and 10 WAP, respectively, than the low P counterparts. Similarly, mycorrhizal roots from plants grown in high P soil contained higher levels of the CHT476 mRNA at most time points than mycorrhizal roots from low P soil.

We also examined the levels of mRNAs homologous to the bean PR4 cDNA clone, which encodes an acidic, extracellular endochitinase (Margis-Pinheiro *et al.* 1991). The PR4 endochitinase is induced by mercuric chloride treatment and alfalfa mosaic virus infection in leaves. The PR4-deduced amino acid sequence is only 41% homologous to the CHT476/CH5B encoded isoform, and the PR4 and CHT476/CH5B probes do not cross-hybridize as determined from different genomic Southern patterns (Broglie *et al.* 1986; Margis-Pinheiro *et al.* 1991).

At the low P concentration, PR4 transcripts accumu-

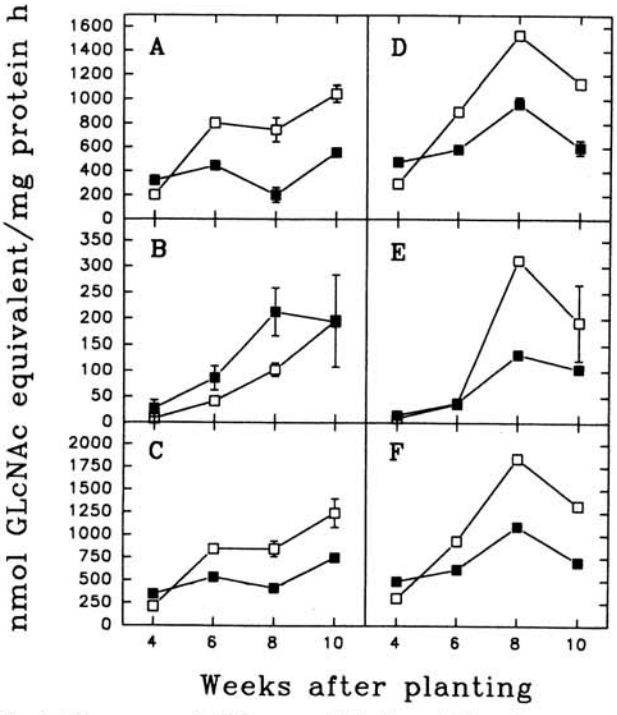

Fig. 1. Time course of chitinase activities in noninfected bean roots (□) or bean roots infected with *Glomus intraradices* strain 25 (■). Activities of endochitinase (**A**), exochitinase (**B**), and total chitinase (**C**) in roots of plants grown in low-phosphate soil and of endochitinase (**D**), exochitinase (**E**), and total chitinase (**F**) in roots of plants grown in high-phosphate soil. Data are expressed in nanomoles of GlcNAc equivalent per milligram of protein per hour, and are the average of two sets of five individual plant roots ± standard deviation of the mean.

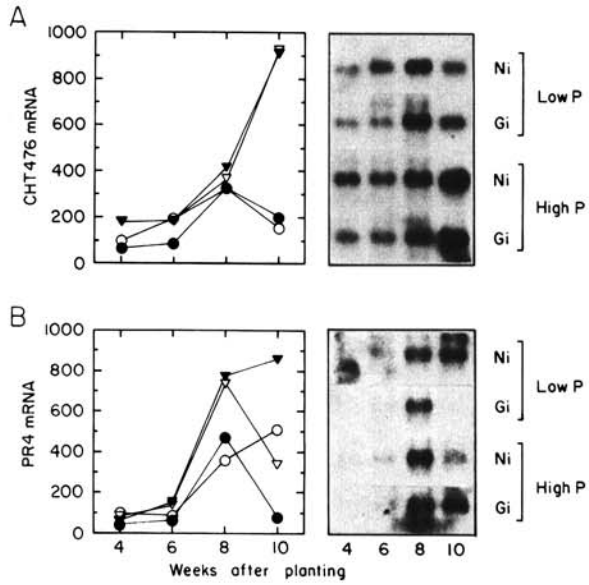

Fig. 2. Steady-state levels of chitinase mRNAs in noninfected (○, ▽) bean roots or roots infected with *Glomus intraradices* strain 25 (●, ▼). Data from plants grown at the low phosphate concentration are represented by circles and at the high phosphate concentration by triangles. The graphs represent the quantification in relative units of the autoradiograms shown at right. Poly(A)$^+$ RNA (2 μg per lane) was analyzed by Northern blotting. **A,** The blot was hybridized with a ^{32}P-labeled CHT476 probe. **B,** The same blot used for CHT476 hybridization was washed and rehybridized with a ^{32}P-labeled PR4 probe.

lated in noninfected roots up to 10 WAP (Fig. 2B). Mycorrhizal roots contained 2.3-, 1.4-, and 6.5-fold less PR4 mRNA at 4, 6, and 10 WAP, respectively, than noninfected controls at the same time points. The PR4 transcript was present at a slightly higher level (1.3-fold) at 8 WAP in mycorrhizal roots.

In noninfected roots from plants grown in high P soil, the PR4 mRNA level was maximal at 8 WAP and then declined to a lower level. The PR4 mRNA in mycorrhizal roots showed a pattern that was similar overall, except that the mRNA level continued to increase up to 10 WAP such that there was a 2.5-fold higher level than in noninfected roots at this time.

β-1,3-Glucanase activities.

β-1,3-Endoglucanase activity and mRNAs have been often shown to be coregulated with endochitinase. To determine whether β-1,3-endoglucanase shows a similar pattern of suppression as endochitinase, we examined endo-, exo-, and total β-1,3-glucanase activities in noninfected and mycorrhizal roots grown at the low and high P concentration. Figure 3 shows that at the low P concentration, β-1,3-endoglucanase specific activity in mycorrhizal roots was 44% and 37% lower than in noninfected roots at 6 and 10 WAP, respectively (Fig. 3A). These differences were significant, whereas the differences at other times were not (t test, $P = 0.05$). In contrast, β-1,3-exoglucanase activity was significantly higher at 4 WAP in mycorrhizal roots (Fig. 3B). The pattern of total glucanase activity primarily reflects the contribution of β-1,3-endoglucanase activity, which constitutes 72–97% of the total activity at different times (Fig. 3C).

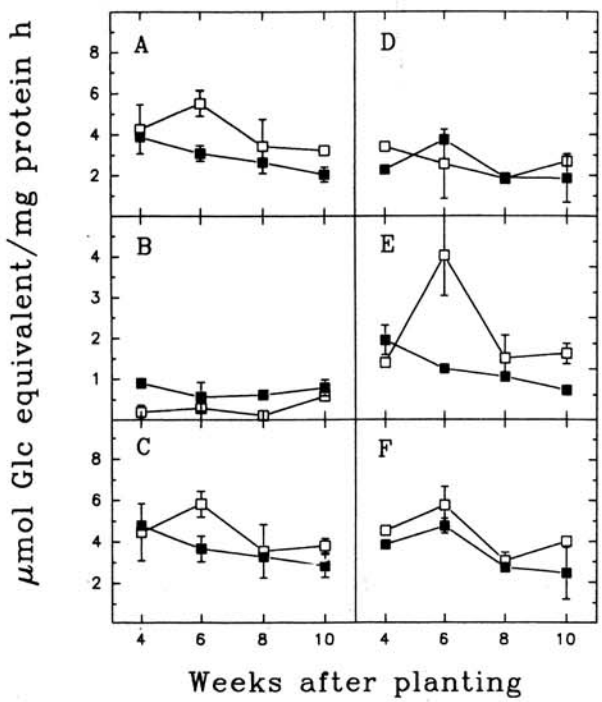

Fig. 3. Time course of β-1,3-glucanase activities in noninfected bean roots (□) and in roots infected with *Glomus intraradices* strain 25 (■). Activities of β-1,3-endoglucanase (**A**), β-1,3-exoglucanase (**B**), and total β-1,3-glucanase (**C**) in roots of plants grown in low-phosphate soil, and of β-1,3-endoglucanase (**D**), β-1,3-exoglucanase (**E**), and total β-1,3-glucanase (**F**) in roots of plants grown in high-phosphate soil. Data are expressed in micromoles of glucose equivalent per milligram of protein per hour, and are the average of two sets of five individual plant roots ± standard deviation of the mean.

At the high P concentration, β-1,3-endoglucanase specific activity in mycorrhizal roots was 33% lower than in noninfected roots at 4 WAP, which was significant (t test, $P = 0.05$; Fig. 3D). In contrast to the results obtained at the low P concentration, exoglucanase activity was lower in mycorrhizal roots at 6 and 10 WAP than in noninfected roots (Fig. 3E). Total glucanase activities are presented in Figure 3F.

In order to determine if the suppression of β-1,3-endoglucanase activity in mycorrhizal roots was due to the presence of an enzyme activity inhibitor, we performed mixing experiments. Equal volumes of crude enzyme extracts from mycorrhizal and noninfected roots of plants grown at the low and high P concentrations were mixed, and β-1,3-glucanase activities were determined. The predicted activities were not significantly different from the measured activities (data not shown). These data indicate that the decreases in β-1,3-endoglucanase activities in mycorrhizal roots under both P conditions were not due to the presence of inhibitors.

β-1,3-Endoglucanase mRNAs.

To determine whether changes in β-1,3-endoglucanase activities were correlated with changes in β-1,3-endoglucanase mRNA levels, we examined the levels of mRNAs encoding two different isoforms. A soybean cDNA clone designated EG488 encodes a soybean basic β-1,3-endoglucanase whose mRNA is induced by ethylene in soybean hypocotyls (Takeuchi *et al.* 1990). Under hybridization conditions allowing no more than 10–15% mismatch, this probe hybridized to two bands in bean genomic DNA digested with restriction enzymes that do not cut the soybean cDNA clone (data not shown).

In Northern blot analysis of bean poly(A)$^+$ RNA with the EG488 probe, a single 1.5-kb mRNA of a size identical to that of the soybean EG488 mRNA was hybridized (Takeuchi *et al.* 1990). At the low P concentration, the level of EG488 hybridizing mRNA in noninfected plants was highly regulated during development, with maximum levels at 8 WAP (Fig. 4A). In contrast, mycorrhizal roots showed a suppressed developmental accumulation of the EG488 hybridizing transcript. At 6 and 8 WAP, the EG488-hybridizing mRNA in mycorrhizal roots was reduced 5.4- and eightfold, respectively, compared with noninfected roots.

At the high P concentration, noninfected roots showed a similar pattern of EG488-hybridizing mRNA regulation as noninfected low P roots except that the mRNA levels were reduced (Fig. 4A). The levels at 6 and 8 WAP were suppressed 5.2- and 5.6-fold, respectively, compared with the equivalent samples from plants grown at the low P concentration. The EG488-hybridizing transcript was further reduced in high P mycorrhizal roots (11.5-fold at 8 WAP), compared with high P noninfected roots.

The regulation of a second β-1,3-endoglucanase mRNA during mycorrhizal development was also analyzed (Fig. 4B). The G101 mRNA encodes a fungal elicitor-induced

bean basic β-1,3-endoglucanase (Edington *et al.* 1991). The G101 and EG488 sequences have very low homology (26% at the nucleotide level and 33% at the amino acid level). Lack of cross-hybridization between the probes was confirmed by different patterns of hybridization on genomic Southern blots (data not shown).

The G101 probe hybridized to the expected 1.4-kb mRNA (Edington *et al.* 1991). In noninfected plants at the low P concentration, the G101 mRNA showed a distinctly different pattern of developmental regulation than the EG488-hybridizing mRNA (Fig. 4A, B). The mRNA accumulated to maximal levels at 10 WAP. As had been observed for the EG488 mRNA, the levels of G101 transcripts were suppressed in mycorrhizal roots compared with noninfected roots at several times, especially at 10 WAP (13-fold suppression).

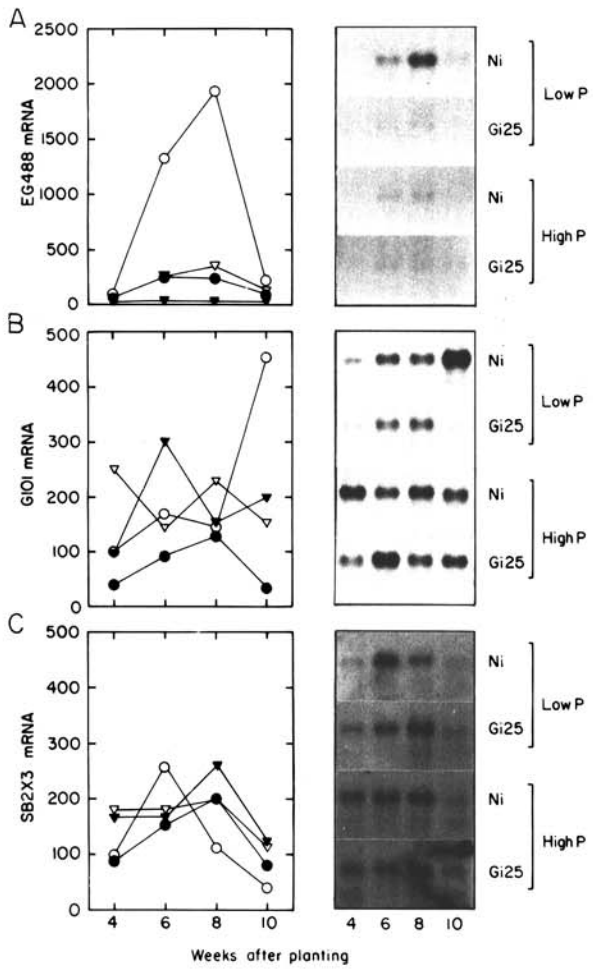

Fig. 4. Steady-state levels of β-1,3-endoglucanase and β-tubulin mRNAs in noninfected (○, ▽) bean roots and roots infected with *Glomus intraradices* strain 25 (●, ▼). Data from plants grown at the low phosphate concentration are represented by circles and at the high phosphate concentration by triangles. The graphs represent the quantification in relative units of the autoradiograms shown at right. Poly(A)$^+$ RNA (2 μg per lane) was analyzed by Northern blotting. **A,** The blot was hybridized with a ^{32}P-labeled EG488 probe. **B,** An identically prepared blot was hybridized with a ^{32}P-labeled G101 probe. **C,** The same blot used for EG488 hybridization was washed and rehybridized with a ^{32}P-labeled SB2X3 probe. Ni = noninfected, and Gi25 = infected with *Glomus intraradices* strain 25.

At the high P concentration, noninfected roots contained moderate, relatively uniform levels of the G101 mRNA throughout the time course in contrast to the accumulation of mRNA observed at the low P concentration. In mycorrhizal roots, the G101 mRNA was reduced at 4 WAP (2.6-fold) and elevated at 6 WAP (2.1-fold) relative to noninfected root levels.

To determine whether an mRNA unrelated to plant defense shows the same or different regulation as the endochitinases and β-1,3-endoglucanase mRNAs, we used a soybean β-tubulin probe (SB2X3), which constitutes a very conserved region of the third exon of the β-tubulin *sb-2* gene and has been shown to hybridize most β-tubulin genes (Han *et al.* 1991). Figure 4C shows that at all weeks after planting the levels of β-tubulin mRNAs were generally comparable in mycorrhizal and noninfected roots at both P concentrations. It can also be observed that the pattern of β-tubulin variation is different from endochitinases and β-1,3-endoglucanases, indicating the uniqueness of their regulation.

Phenylpropanoid pathway mRNA regulation.

The expression of three mRNAs encoding enzymes in the phenylpropanoid pathway were differentially regulated in VA mycorrhiza. Phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI) catalyze reactions necessary for the synthesis of both isoflavonoid phytoalexins and flavonoids. Figure 5 shows that the level of CHI mRNA was lower in mycorrhizal roots than in noninfected roots at all times under both low and high soil P conditions. Under low P concentration, the levels of CHI mRNA in mycorrhizal roots were approximately 1.3-fold lower than in noninfected roots at all weeks, whereas under high P concentration mycorrhizal roots contained 2.4-, 1.3-, and 2.1-fold less CHI mRNA than noninfected roots at 4, 6, and 8 WAP, respectively. In addition, it is interesting to note that the levels of CHI mRNA were generally higher (1.2- to 3.9-fold) in both noninfected and mycorrhizal plants grown in high P soil than in the low P counterparts. The levels of mRNAs encoding PAL and CHS in mycorrhizal roots were comparable to noninfected roots at the low and high P concentrations (data not shown).

DISCUSSION

We observed that under the low and high P concentrations, endochitinase specific activity was higher in

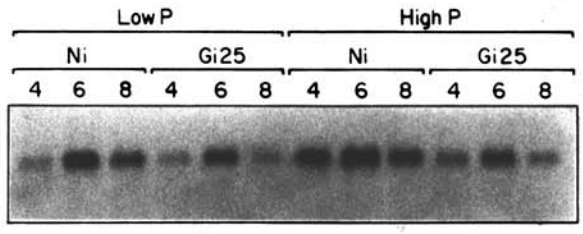

Fig. 5. Steady-state levels of chalcone isomerase mRNA in noninfected (Ni) bean roots and roots infected with *Glomus intraradices* strain 25 (Gi25) grown at low and high phosphate concentrations. A blot prepared identically to the one described in Fig. 2 was hybridized with a ^{32}P-labeled chalcone isomerase probe.

mycorrhizal roots than in noninfected roots at an early stage of root colonization followed by suppression of activity at later stages. Suppression of β-1,3-endoglucanase activity and mRNAs occurred at specific stages during mycorrhiza development under both low and high P concentrations. Our experimental approaches did not permit discrimination between plant and fungal chitinase and β-1,3-glucanase activities. However, if the fungus contributes to these activities in mycorrhizal roots then the suppression of plant endochitinase and β-1,3-endoglucanase activities observed would be even greater than indicated by the observed changes in the total activities. In addition, mycorrhizal roots were shown to contain either the same or reduced levels of PAL, CHS, and CHI mRNAs.

An important consideration in our experimental design was to ensure that the physiological status of mycorrhizal and noninfected plants were comparable. Noninfected plants were provided with extra supplements of nutrients, which resulted in comparable shoot and root biomasses in mycorrhizal and noninfected plants. The approximate equivalence in growth in the two sets of plants allowed us to make clear comparisons based on the presence or absence of mycorrhiza.

The initial increase in endochitinase specific activity followed by suppression in bean mycorrhizal roots grown at the low and high P concentration is similar to the kinetics previously reported for chitinase activity in *Allium porrum* infected by *Glomus versiforme* (Spanu *et al.* 1989). Furthermore, the suppression of chitinase activity in leek mycorrhizal roots is not due to the presence of an inhibitor, and we also excluded this possibility for bean mycorrhizal roots. In contrast, Dumas-Gaudot *et al.* (1992) reported increased activity of specific chitinase isoforms in leek, onion, and pea infected by different *Glomus* species, even during the late stages of VA mycorrhiza development.

Although the patterns of endochitinase activity in mycorrhizal roots at the low and high P concentrations were similar, we found that endochitinase activity was suppressed to a greater extent at low P. Under low P, the greatest suppression (from 1.8- to 3.7-fold) was observed between 6 and 8 WAP. This period of maximal suppression coincided with the period of highest fungal growth rate (8.1 cm infected root/day). These data suggest that endochitinase activity may limit the intraradical growth of mycorrhizal fungi. At the high P concentration, suppression of endochitinase activity was smaller (1.5- to 1.9-fold, between 6 and 10 WAP), and the remaining higher levels of endochitinase activity in mycorrhizal roots may contribute to the reduced fungal growth rates observed at this P concentration.

We monitored the expression of two bean mRNAs encoding basic and acidic endochitinase isoforms to assess the correlation between the levels of these mRNAs and the observed suppression of chitinase activity. At the low soil P concentration, the CHT476 basic isoform mRNA was reduced at 4 and 6 WAP in mycorrhizal roots but not in the later weeks. The PR4 acidic isoform mRNA was markedly reduced at 10 WAP but not at earlier times. We propose from these data that the observed reductions in both mRNA species contribute to the reduced endochitinase activities in mycorrhizal roots at the low P concentration. This possibility will need to be verified by resolving the different isoforms so that specific isoform-mRNA correlations can be made. A number of different chitinase isoforms have been characterized in bean leaves (Vögeli *et al.* 1988; Awade *et al.* 1989; Margis-Pinheiro *et al.* 1991), but the relative expression of isoforms in roots is entirely unknown. There is considerable precedent for differential regulation of members of chitinase gene families by environmental and developmental factors (Benhamou *et al.* 1990; Herget *et al.* 1990).

The functional role of endochitinases during the development of VA mycorrhiza is unclear. There is increasing evidence that endochitinase is important in containing plant pathogen infections by virtue of its ability to degrade fungal cell walls (Mauch *et al.* 1988; Toyoda *et al.* 1991). Spanu *et al.* (1989) showed that plant chitinase does not bind external hyphae of a VA mycorrhizal fungus unless fungal cell wall proteins and soluble carbohydrates are removed by a heat treatment, and they suggested that chitinase per se could not degrade VA mycorrhizal fungus hyphae. In addition, their immunolocalization studies indicated that chitinase is not associated with fungal structures, with the exception of senescing arbuscules. However, this study did not directly examine growing hyphal tips, where chitin is more exposed (Wessels 1990) and which is the known site of plant chitinase antifungal action (Mauch *et al.* 1988). In addition, immunologically unrelated chitinases might be present that would be unrecognized by the bean monospecific antibody used in the study. Although there is no information on the molecular mechanisms controlling VA mycorrhiza development, suppression of plant endochitinases may facilitate mycorrhizal fungus growth. An additional role might be to degrade collapsing arbuscules and old intercellular hypha. Endochitinase per se may not be the only factor controlling VA mycorrhiza development at low and high P concentrations, because other defense-related genes, such as β-1,3-endoglucanases and CHI, are being differentially regulated during mycorrhiza development.

β-1,3-Endoglucanases are part of a well-characterized group of pathogenesis-related proteins that are induced by a variety of biotic and abiotic factors (Carr and Klessig 1990). It has been observed that β-1,3-endoglucanases and endochitinases are coregulated in bean leaves (Vögeli *et al.* 1988). However, in our system, the patterns of endochitinase and β-1,3-endoglucanase expression were distinct. Several β-1,3-endoglucanase isoforms are expressed in bean under different environmental conditions. Edington *et al.* (1991) proposed that the G101 mRNA encodes the major basic β-1,3-endoglucanase of bean in leaves (Mauch and Staehelin 1989). Two minor extracellular acidic isoforms have also been detected in bean leaves after mercuric chloride treatment or viral infection (Awade *et al.* 1989). Interestingly, the soybean EG488 cDNA clone shows highest homology (63% at the amino acid level) to an acidic tobacco glucanase cDNA clone, PR-Q′ (Payne *et al.* 1990). It is possible that the EG488-hybridizing mRNA in bean also encodes an acidic glucanase, since only one basic isoform, presumably encoded by the G101 mRNA, has been observed under

various stress conditions (Vögeli *et al.* 1988; Awade *et al.* 1989; Mauch and Staehelin 1989).

In mycorrhizal plants grown at the low P concentration, suppression of β-1,3-endoglucanase activity at 6 and 10 WAP was associated with marked reductions in the levels of both EG488 and G101 mRNAs. At the high P concentration, β-1,3-endoglucanase activity was reduced at 4 WAP compared with noninfected roots. While the EG488-hybridizing mRNA was present at comparable levels in mycorrhizal and noninfected roots at this time, the G101 mRNA was suppressed only at 4 WAP in mycorrhizal roots and may be the mRNA primarily affecting glucanase activity at the high P concentration. The levels of β-1,3-endoglucanase activity in noninfected and mycorrhizal roots at the high P concentration were comparable to the levels at the low P concentration.

It is not likely that β-1,3-endoglucanases control VA mycorrhizal fungi growth by degrading the fungal cell wall as proposed for plant-pathogen interactions, since β-1,3-glucans are absent from the cell walls of the Zygomycetes (Wessels 1990). It is more likely that β-1,3-endoglucanase expression is modulated as part of the general defense response, as in the case of its regulation during viral infection where a substrate for the enzyme is also not clearly present (Carr and Klessig 1990). In addition, it is unlikely that the observed suppression in accumulation of only CHI mRNA, and not PAL and CHS mRNAs, would be a factor resulting in reduced flavonoid or isoflavonoid phytoalexin synthesis in mycorrhizal roots. In soybean mycorrhizal roots, a very limited accumulation of phytoalexins occurs late during the infection process (Morandi *et al.* 1984; Wyss *et al.* 1991).

The mechanisms by which VA mycorrhizal fungi regulate expression of endochitinase and β-1,3-endoglucanase are unknown. Several lines of evidence indicate that VA mycorrhizal fungi may alter the levels of cytokinins and gibberellinlike compounds in roots (Allen *et al.* 1980; Allen *et al.* 1982). In addition, McArthur and Knowles (1991) have found that potato mycorrhizal roots are depressed in ethylene production and the ability to convert 1-aminocyclopropane-1-carboxylic acid to ethylene. Chitinase and glucanase activity and mRNA accumulation were shown to be suppressed in cultured tobacco tissues by combinations of auxin and cytokinins at physiological concentrations, despite high levels of ethylene production (Mohnen *et al.* 1985; Felix and Meins 1986, 1987; Shinshi *et al.* 1987). The CHT476/CH5B endochitinase transcript is induced by ethylene in bean leaves (Broglie *et al.* 1986) as well as the major basic bean glucanase mRNA (Vögeli *et al.* 1988). In addition, the EG488 β-1,3-endoglucanase transcript is induced by ethylene in soybean cotyledons (Yoshikawa *et al.* 1990). It remains to be determined whether these transcripts are ethylene-regulated in roots.

From the studies described above, we suggest that suppression of endochitinase and β-1,3-endoglucanase activities in bean VA mycorrhizal roots may result from 1) alterations in the auxin and cytokinin balance that affects chitinase and glucanase expression by reduced ethylene production and/or an ethylene-independent mechanism or from 2) direct suppression of ethylene production. In addition, the suppression of one of the β-1,3-endoglucanase transcripts by high concentrations of P in the soil may also involve hormonal changes, since P has been shown to affect cytokinin levels in root exudate of sycamore seedlings (Dhillon 1978) and increase ethylene production in potato roots (McArthur and Knowles 1991).

Another possibility to consider is that VA mycorrhizal fungi may produce a nonhormone suppressor of chitinase and glucanase expression. Glucans and glycopeptides isolated from pathogenic fungi have been shown to suppress the hypersensitive response and phytoalexin accumulation (Shiraishi *et al.* 1978; Doke *et al.* 1979).

In conclusion, our study demonstrated suppressed levels of several defense-related mRNAs in bean VA mycorrhizal roots. The results of this study suggest that VA mycorrhizal fungi are able to suppress the general plant defense mechanism under both low and high P concentrations. Under high P concentration, the lower level of endochitinase suppression may contribute to the reduced root colonization. Future studies using transgenic plants overexpressing sense or antisense chitinase or β-1,3-glucanase genes will provide valuable systems to directly test the involvement of these hydrolases on mycorrhiza development. In addition, elucidation of changes in hormone levels in mycorrhizal roots is critical for investigation of their possible involvement in the suppression of chitinase and other defense-related genes.

MATERIALS AND METHODS

Fungal strain.

Glomus intraradices Shenck & Smith strain 25 was obtained from Native Plants, Inc. (Salt Lake City, UT). Spores were surface-sterilized in 0.5% NaOCl and 200 $\mu g \cdot ml^{-1}$ streptomycin for 5 min and washed thoroughly with sterile tap water.

Plant material, inoculation, and growth.

Bean (*Phaseolus vulgaris* L. 'Tendergreen') seeds were surface-sterilized with 1.58% NaOCl for 20 min and pregerminated on sterile, wet filter paper at 27° C. Plants were grown in a steam-sterilized (121° C/3 h, 2 cycles) mixture of river sand and peat moss (2:1, v/v), pH 6.5 (in H_2O), inoculated with 10 ml of a spore suspension (200 spores $\cdot ml^{-1}$) of *G. intraradices* strain 25. Control pots were inoculated with 10 ml of a spore suspension filtrate. Plants (one per pot) were grown at 27° C day/20° C night, 60% relative humidity, and 12-h photoperiod. Noninfected plants grown in low P soil (20 mg P per kilogram of soil) were supplemented with 72 mg N, 74 mg K, 50 mg Ca, 35 mg Mg, 2 mg Fe, and 0.2 ml/pot of Hoagland's micronutrient solution. Mycorrhizal plants received approximately 75% of these levels in order to obtain plants with approximately the same growth characteristics. All plants grown under high P concentration (150 mg P per kilogram of soil) received 88 mg N, 165 mg K, 65 mg Ca, 45 mg Mg, 3 mg Fe, and 0.3 ml/pot of Hoagland's micronutrient solution. Ten plants per treatment were harvested at 4, 6, 8, and 10 WAP. Shoots were dried and weighed. Roots were washed, weighed, fixed in FAA (37% formaldehyde, 50% ethanol, and 5% glacial acetic acid in water), and stained with trypan blue for determination of the infection

level and total root length by the intersection method (Giovanetti and Mosse 1980). The remaining roots from individual plants were frozen separately in liquid nitrogen and stored at −70° C.

Protein extraction.

Individual frozen root samples were combined (equal amounts from five bean plants) in order to have two repetitions of each treatment. Proteins were extracted as previously described with modifications (Vögeli *et al.* 1988). Combined samples were homogenized in 0.1 M sodium citrate buffer pH 5.0 (3 ml per gram of frozen roots) containing 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged twice at 10,000 × *g* for 20 min, filtered through a 0.45-μm membrane filter, and brought to 95% saturation at 0° C with solid $(NH_4)_2SO_4$. After 2 h on ice, pellets were collected by centrifugation at 10,000 × *g* for 20 min, resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 10 mM 2-mercaptoethanol, and dialyzed against the same buffer at 3° C. The resultant crude enzyme extract was stored at −20° C. Protein concentration was determined in duplicates using the standard protocol for the bicinchonic acid protein assay reagent (Pierce, Rockford, IL) and bovine albumin as the standard.

Chitinase and β-1,3-glucanase assays.

Exochitinase and endochitinase activities were assayed colorimetrically by the release of *N*-acetylglucosamine from colloidal chitin as described by Boller *et al.* (1983).

Total β-1,3-glucanase enzyme activity was assayed by the release of reducing sugars from laminarin (Sigma, St. Louis, MO), as described by Keen and Yoshikawa (1983). β-1,3-Exoglucanase activity was assayed by the release of glucose from laminarin. Glucose concentration was determined with the glucose hexokinase reagent, according to the manufacturer's instructions (Sigma, St. Louis, MO).

RNA analysis.

Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Maniatis *et al.* 1982) and size-fractionated on formaldehyde-agarose gels. Equal RNA loading was based on spectrophotometric determination of RNA concentration and ethidium bromide staining. RNA was transferred to Duralon-UV membranes and hybridized according to the manufacturer's instructions (Stratagene, La Jolla, CA) with a ^{32}P-labeled DNA probe for 24–36 h at 60° C for homologous probes and 50° C for heterologous probes. Membranes were washed for 30 min with either 0.1× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 1% SDS or 0.5× SSPE, 1% SDS, for homologous and heterologous probes, respectively, at the same temperature used for hybridization. Densitometric analyses of autoradiograms were carried out using an Isco gel scanner model 1312 (Isco, Lincoln, NE).

DNA probes.

DNA was ^{32}P-labeled using the random primer method. CHT476 probe (basic bean endochitinase) is a 1,100-bp *Eco*RI fragment of the plasmid pCHT476 (Sheng and Mehdy, unpublished). PR4 probe (bean acidic endochitinase) is a 976-bp *Eco*RI fragment of the plasmid pKSPR4 (Margis-Pinheiro *et al.* 1991). EG488 probe (soybean basic β-1,3-endoglucanase) is a 1,257-bp *Eco*RI fragment of the plasmid pEG488 (Takeuchi *et al.* 1990). G101 probe (bean basic β-1,3-endoglucanase) is a 923-bp *Pst*I fragment of the plasmid pG101 (Edington *et al.* 1991). SB2X3 probe (soybean β-tubulin) is a 550-bp *Eco*RI-*Hin*dIII fragment of the plasmid pSB2X3 (Han *et al.* 1991). CHI probe (bean chalcone isomerase) is a ~865-bp *Eco*RI fragment of the plasmid pCHI1 (Mehdy and Lamb 1987).

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