Isoflavonoid Accumulation and Expression of Defense Gene Transcripts During the Establishment of Vesicular-Arbuscular Mycorrhizal Associations in Roots of *Medicago truncatula*

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The levels of flavonoid and isoflavonoid secondary metabolites and mRNA transcripts encoding enzymes of their biosynthesis were measured in *Medicago truncatula* and *Medicago sativa* roots during colonization with the vesicular-arbuscular mycorrhizal fungus *Glomus versiforme*. Distinct qualitative and quantitative changes in secondary metabolites occurred during the establishment of the symbiosis, including transient increases in the level of the phytoalexin medicarpin during the early stages of colonization. Levels of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) transcripts were elevated in mycorrhizal roots, consistent with increased flavonoid biosynthesis. In contrast, isoflavone reductase (IFR) transcripts, which encode an enzyme specific for medicarpin biosynthesis, decreased below the levels in control roots during the later stages of the interaction. The decrease in IFR transcripts, and the subsequent decrease in medicarpin levels, did not occur in unsuccessful interactions between *G. versiforme* and a mycorrhizal minus (myc$^-$) line of *M. sativa*.

Additional keywords: Glomus versiforme, Medicago sativa, Medicago truncatula, isoflavone reductase, medicarpin, symbiosis.

Mycorrhizae are widespread symbiotic associations that form between terrestrial plants and certain groups of fungi. Vesicular-arbuscular (VA) mycorrhizae are the most frequently occurring type of mycorrhizal association of agricultural crop species and may be significantly beneficial to plant growth and nutrition (Jeffries 1987). The fungal partner grows predominantly inside the root but also contributes a network of fine hyphae external to the root, which increases the absorption and translocation of limiting nutrients (especially phosphorous) from the soil to the plant. In exchange, the fungus, an obligate biotroph, obtains carbohydrate from the plant. This bi-directional exchange of nutrients occurs through extensively branched haustoria called arbuscules. In addition to increased nutrition, mycorrhizal plants also show increased resistance to root pathogens, including nematodes, and increased tolerance to drought stress (Jeffries 1987; Linderman 1988; Smith 1988; Hwang *et al.* 1992).

The process of colonization by the fungus and establishment of an active association involves a complex sequence of interactions between the fungal hyphae and the plant root cells. These include contact between fungal hyphae and the root surface, differentiation to form appressoria, penetration and hyphal growth within the root, and further differentiation to specialized structures involved in nutrient exchange. The process has been clearly documented microscopically (Brundrett *et al.* 1985) and physiologically (Smith and Gianinazzi-Pearson 1988); however, very little is known of the biochemical and molecular genetic processes involved.

Both the differentiated structures associated with the symbiosis and the plant's reaction to colonization are somewhat reminiscent of the early stages of biotrophic or hemibiotrophic compatible fungal plant-pathogen interactions, where the pathogen successfully invades large areas of the host and initially avoids eliciting a host defense response (Dixon and Harrison 1990). Very low levels of glyceollin (the major phytoalexin of soybean), and the related isoflavonoids daidzein and coumestrol have, however, been reported to accumulate in fully established mycorrhizae in soybean (Morandi *et al.* 1984), although glyceollin was not detected in the early stages of the association (Wyss *et al.* 1991).

Recently, attempts have been made to identify plant compounds that may act as signal molecules for the initiation and maintenance of mycorrhizal interactions. By analogy to the *Rhizobium*–legume symbiosis and to plant-parasitic associations (reviewed in Lynn and Chang

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1990), this work has focused on various flavonoid derivatives. The results suggest that a variety of flavanones, flavones, and isoflavones are capable of stimulating spore germination and hyphal growth of VA fungi *in vitro* (Gianinazzi-Pearson *et al.* 1989; Tsai and Phillips 1991; Nair *et al.* 1991); however, in an elevated CO_2 atmosphere it was reported that among the flavonoids tested only the flavonols stimulated fungal growth, while the other derivatives were all inhibitory (Bécard *et al.* 1992). Although the effects of various flavonoid/isoflavonoid metabolites on fungal growth have been studied *in vitro*, the changes in the profiles of such metabolites in roots undergoing the formation of a symbiotic association have not been examined.

In legumes such as alfalfa, antimicrobial phytoalexins, inducers of rhizobial nodulation genes, and the flavonoid derivatives shown to stimulate mycorrhizal growth are all biosynthetically related (Dixon *et al.* 1992). We have therefore initiated a detailed analysis of flavonoid/isoflavonoid metabolites in roots of *Medicago truncatula*, a genetically tractable model legume for the study of rhizobium–legume symbiosis (Barker *et al.* 1990), to determine whether the mycorrhizal interaction involves changes in the balance of potentially inhibitory or stimulatory compounds. We have complemented this approach by analysis of the levels of transcripts encoding L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the first committed step in the phenylpropanoid pathway; chalcone synthase (CHS, EC 2.3.1.74), the first enzyme specific for flavonoid/isoflavonoid biosynthesis; and isoflavone reductase, the penultimate enzyme in the biosynthesis of the phytoalexin medicarpin and thus specific for antimicrobial isoflavonoid metabolism (Paiva *et al.* 1991). In addition to *M. truncatula,* we have also included analysis of a mycorrhizal minus (myc^-) line of a closely related species, *M. sativa.*

Our results reveal qualitative and quantitative changes in the flavonoid profiles of roots undergoing development of a mycorrhizal association, including the appearance of some of the compounds previously suggested to influence VA fungal growth. In addition, we observe a transient increase in isoflavonoid phytoalexins in the early stages of the interaction. IFR transcripts, which are generally coordinately regulated with PAL and CHS to effect phytoalexin biosynthesis, specifically decrease in the established symbiosis. In contrast, IFR transcripts remain elevated in the interaction between a VA fungus and a myc^- line of *M. sativa,* which is unable to form a complete association.

RESULTS

Colonization of *M. truncatula* with *G. versiforme*.

G. versiforme rapidly colonized roots of *M. truncatula* with a rate of infection similar to that reported for colonization of *M. sativa* (Brundrett *et al.* 1985). As a mixed inoculum consisting of spores, hyphae, and colonized root pieces was used, the infection process is not synchronous and even at the later stages of colonization there may be initiation of new infections from spores or external hyphae. It was therefore necessary to consider carefully which time points to analyze. By 7 days after inoculation numerous external hyphae, internal hyphae, and arbuscules were present; however, although the length of root colonized was quite high (30%), there was generally only one row of cortical cells that contained arbuscules. By 13 days after inoculation the amount of colonization had increased to 70% of the root length and vesicles were also abundant. By 40 days, colonization was extensive (>75%) and in many sections of the root the majority of the cortical cells contained arbuscules (Fig. 1A, B). The above time points were therefore chosen to represent stages of colonization as observed by morphological criteria. The first two time points represent two stages of development of the association, while the third point (40 days after colonization) represents an older fully established association. The choice of these time points was supported by previous observations in *Allium porrum* colonized with *G. versiforme* where chitinase and cell wall-bound peroxidase activities show most significant changes between 10 and 20 days after colonization, with few changes occurring between 30 and 90 days when the interaction is fully established (Spanu and Bonfante-Fasolo 1988; Spanu *et al.* 1989).

Flavonoid/isoflavonoid profiles of mycorrhizal root extracts.

A comparison of the flavonoid profiles of extracts from control *M. truncatula* roots and from initiating (7 and 13 days after colonization) and well-established (40 days) mycorrhizal associations between *M. truncatula* and *G. versiforme* is show in Figure 2A–D. The flavonoid profiles of extracts from control roots (Fig. 2A) are relatively simple and contain three major peaks, two of which have been identified as formononetin malonyl glucoside (FGM) (L. Sumner, N. Paiva, and R. A. Dixon, unpublished results) and medicarpin malonyl glucoside (MGM) (Kessmann *et al.* 1990b). The third metabolite has a retention time of 37.5 min (peak 37) and is an unknown that appears to be a conjugate on the basis of hydrolysis with β-glucosidase (data not shown). These profiles are very similar to those of root extracts from *M. sativa* and extracts from *M. sativa* cell suspension cultures (Kessman *et al.* 1990a, b). The results in Figure 2 indicate that, as colonization proceeds, there are significant increases in the levels of FGM, MGM, and the isoflavone daidzein. By 40 days after inoculation, the average increases were 3.5-, 4.5-, and 3.1-fold, respectively, as compared to the levels in the uninoculated control root extracts of the same age. The absolute levels of these metabolites in both the controls and colonized roots are represented graphically in Figure 3. FGM, MGM, and daidzein accumulated to 75.5 μg/g fresh weight, 47.0 μg/g fresh weight, and 8.75 μg/g fresh weight, respectively.

In contrast to their conjugated forms, free medicarpin (a major antimicrobial phytoalexin in alfalfa) and its isoflavone precursor formononetin were present at very low or undetectable levels in control root extracts but underwent transient increases in the early stages of colonization. Medicarpin levels increased to 3.44 μg/g fresh weight and formononetin levels to 0.54 μg/g fresh weight at 13 days

postinoculation, but both decreased to almost undetectable levels by 40 days postinoculation (Fig. 3). Two unknown metabolites with retention times of 27.5 and 37.5 min (peaks 27 and 37) also underwent similar transient increases in the early stages of the association.

Among the metabolites appearing in mycorrhizal root extracts that were not present in control roots were 4′,7-dihydroxyflavone, a flavonoid previously demonstrated to enhance spore germination of two *Glomus* species (Tsai and Phillips 1991) and coumestrol, previously reported to accumulate in soybean mycorrhizae (Morandi *et al.* 1984). The two compounds showed slightly different kinetics of accumulation during development of the mycorrhizal association. The level of 4′,7-dihydroxyflavone was maximal at 7 days postinoculation (0.28 μg/g fresh weight) and decreased slightly in the established interaction. The level of coumestrol was maximal at 13 days postinoculation (1 μg/g fresh weight) and was very low compared to the level previously reported for soybean (25 μg/g fresh weight) (Morandi *et al.* 1984).

In addition to the identified metabolites described above, colonization also led to changes in many uniden-

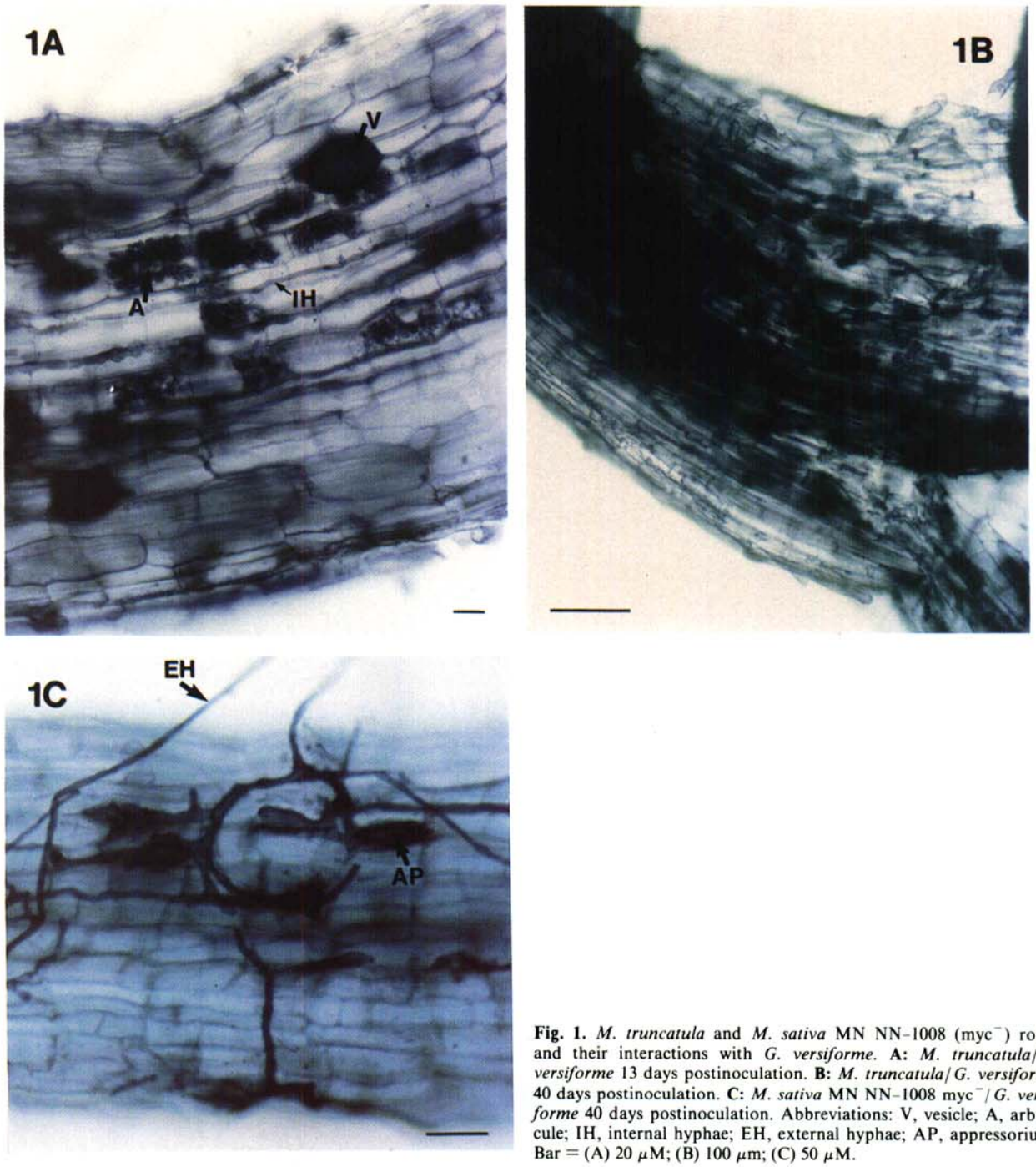

Fig. 1. *M. truncatula* and *M. sativa* MN NN-1008 (myc⁻) roots and their interactions with *G. versiforme*. **A:** *M. truncatula*/*G. versiforme* 13 days postinoculation. **B:** *M. truncatula*/*G. versiforme* 40 days postinoculation. **C:** *M. sativa* MN NN-1008 myc⁻/*G. versiforme* 40 days postinoculation. Abbreviations: V, vesicle; A, arbuscule; IH, internal hyphae; EH, external hyphae; AP, appressorium. Bar = (A) 20 μM; (B) 100 μm; (C) 50 μM.

tified species (Fig. 2A–D). In general, the levels of metabolites revealed by HPLC profiling increased after colonization; however, the levels of at least one metabolite with a retention time of 40 min (peak 40) decreased as colonization proceeded.

M. truncatula and *M. sativa* are closely related, and the levels of flavonoid and isoflavonoid metabolites in *M. sativa* 'Kanza' at 40 days after colonization are very similar to those measured in *M. truncatula* and have been tabulated in Figure 3. As observed with *M. truncatula* MGM, daidzein, coumestrol, and 4′, 7-dihydroxyflavone all accumulate after colonization, and the unidentified metabolite with a retention time of 40 min also decreases after colonization. The major difference between the species is the level of FGM which is considerably higher in *M. sativa* 'Kanza' and does not show a significant increase after colonization. The levels of daidzein and formononetin in *M. sativa* 'Kanza' are also slightly higher

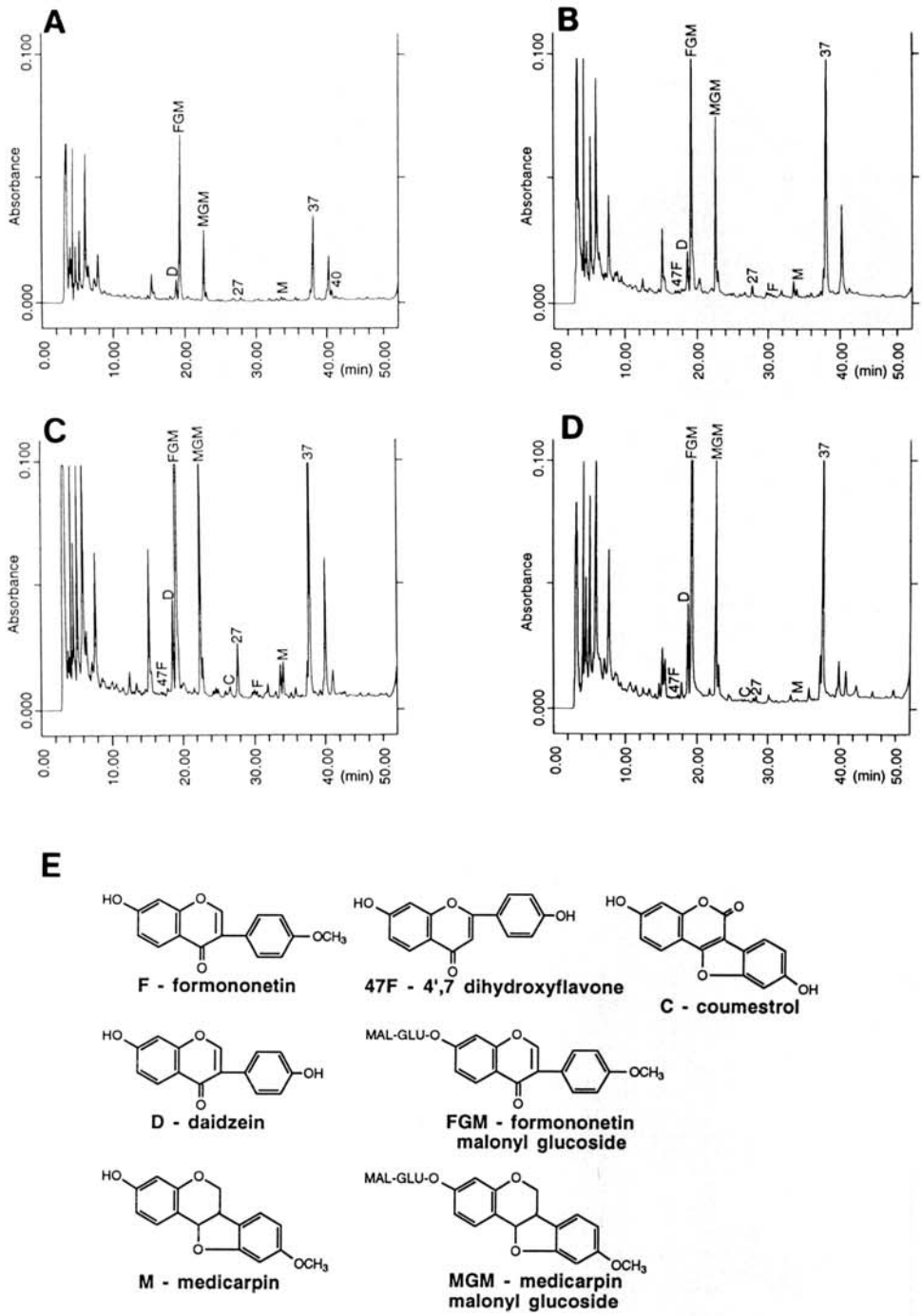

Fig. 2. HPLC profiles of acetone extracts of *M. truncatula* roots. **A:** uninoculated control roots (7 days); **B-D:** roots colonized with *G. versiforme* at 7 **(B),** 13 **(C)** and 40 **(D)** days postinoculation; **E:** structures of the metabolites resolved in **A-D.**

than in *M. truncatula*; however, free formononetin may arise from the breakdown of the FGM conjugate during handling of the root samples.

Expression of PAL, CHS, and IFR transcripts in mycorrhizal interactions.

As significant changes in a number of flavonoid/iso-flavonoid compounds had been observed during establishment of the mycorrhizal association, we proceeded to investigate the transcript levels of three key enzymes involved in the synthesis of these compounds.

The data in Figure 4 and Table 1 indicate that the levels of transcripts encoding PAL and CHS both increased in *M. truncatula* roots during colonization with *G. versiforme*. Transcript levels for PAL increased 1.75 (± 0.25)-fold with colonization (compared to the levels in uninoculated

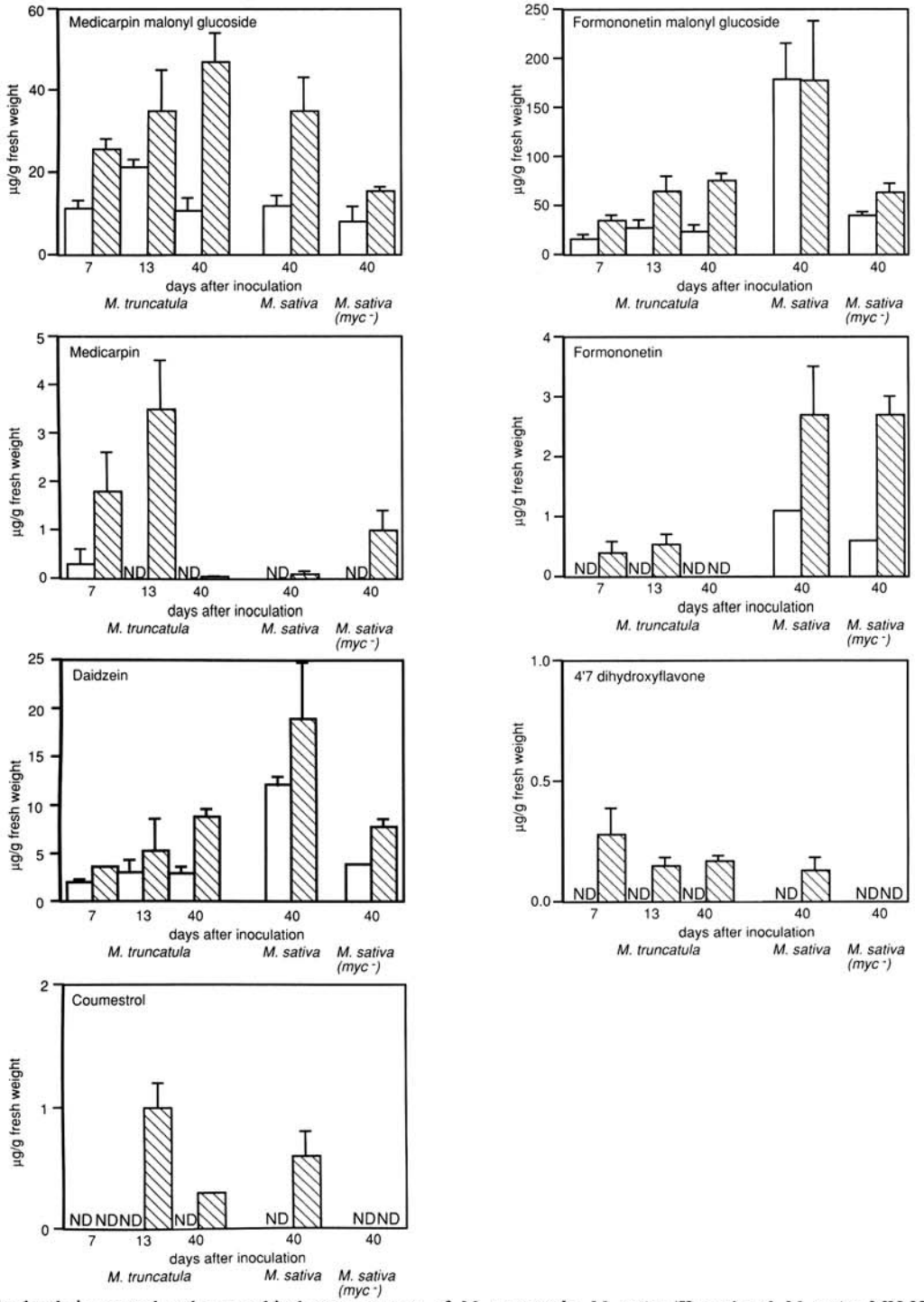

Fig. 3. Metabolite levels in control and mycorrhizal roots extracts of *M. truncatula, M. sativa* 'Kanza' and *M. sativa* MN NN-1008 (myc$^-$) during the development of a mycorrhizal association. □ = metabolite levels in uninoculated control roots; ▨ = metabolite levels in roots colonized with *G. versiforme*. ND = Not detectable. Data are the mean values ± standard deviation for two to four replicates from pooled samples of five to twelve plants.

control roots of the same age), while the level of CHS transcripts was 2.25 (± 0.05)-fold higher than the level in control roots in the initial stages of the interaction and 1.8-fold higher than controls at 40 days postinoculation. In contrast, the level of transcripts encoding IFR decreased as colonization proceeded. Initially, a very slight (possibly not significant) increase in IFR transcript levels was seen at 7 days; however by 13 days, there was a 1.5-fold decrease and by 40 days a 2.5 (± 0.4)-fold decrease below the level in uninoculated control roots (Fig. 4, Table 1). Although these changes are not large, they are consistently reproducible and correlate with the metabolite profiles previously described. Similar increased levels of PAL and CHS transcripts and decreased levels of IFR transcript were observed in *M. sativa* 'Kanza' at 40 days after colonization when compared to the levels of uninoculated controls (Fig. 4 and Table 1).

Effects of fertilization with phosphate.

Many of the reported benefits of colonization by mycorrhizal fungi have been attributed to the increased phosphorous status of the plants. Therefore, to determine whether the changes in flavonoid levels and transcript levels were simply due to the availability of phosphorous, a set of control plants was grown with a phosphate supplement and compared with those grown in the lower phosphate conditions as used in the previous experiments. The flavonoid/isoflavonoid content of the root extracts and levels of transcripts encoding enzymes of phenylpropanoid metabolism were analyzed after 40 days and shown to be the same in both samples (Fig. 5). This indicates that the alterations in flavonoid levels and RNA transcripts that occur in mycorrhizal roots in these experiments are a direct effect of the fungus and not an indirect effect of increased phosphate availability.

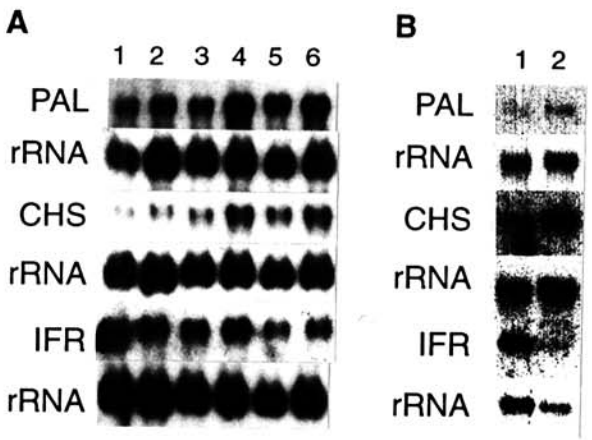

Fig. 4. Changes in the levels of transcripts encoding PAL, CHS and IFR during development of mycorrhizal associations in *M. truncatula* and *M. sativa*. **A.** Northern blot analysis of total RNA (2 μg) from *M. truncatula* uninoculated controls at 7 (lane 1), 13 (lane 2) and 40 (lane 3) days, and 2 μg of total RNA from *M. truncatula* colonized with *G. versiforme* at 7 (lane 4), 13 (lane 5) and 40 (lane 6) days post inoculation. Ribosomal RNA controls for loading and transfer are shown beneath each transcript. **B.** Northern blot analysis of 2 μg of total RNA from *M. sativa* 'Kanza' control (lane 1) and after 40 days of colonization with *G. versiforme* (lane 2).

Characterization of a myc⁻ interaction.

Mutant plants that are unable to form complete mycorrhizae provide a powerful tool with which to further analyze the interaction. Such mutants of *M. truncatula* are not yet available; however, it has previously been observed that a *M. sativa* germplasm (MN NN–1008) altered in its ability to form nodules (nod⁻) is also unable to form a complete mycorrhizal interaction (Bradbury *et al.* 1991). The nod⁻ trait is controlled by two tetrasomically inherited recessive genes (termed nn_1 and nn_2) which are possibly redundant genes that encode the same function (Barnes *et al.* 1988).

The interaction between *M. sativa* MN NN-1008 and *G. versiforme* is blocked at an early stage. Fungal hyphae grow along the surface of the root forming many aberrantly shaped appressoria, but are unable to penetrate the root, and the interaction does not proceed further (Bradbury *et al.* 1991). Thus, by allowing the fungus sufficient time to colonize the surface of the root, it is possible to obtain an amplified view of the earliest stages of the interaction.

MN NN–1008 plants were harvested after 40 days of growth in pots containing fungal inoculum to obtain samples in which many attempted colonization events had occurred. Stained samples of these roots indicated that approximately 80% of the root length had fungal hyphae growing along the root surface. The number of appressoria was higher than that observed in a successful interaction (*M. truncatula/G. versiforme* or *M. sativa* myc⁺/*G. versiforme*) and as reported by Bradbury *et al.* 1991; the fungus did not penetrate the root and the interaction did not proceed further (Fig. 1C). Internal structures were not observed.

Table 1. Relative changes in the transcript levels of PAL, CHS, and IFR after colonization with *G. versiforme*

	Fold changes in transcript level over noncolonized root[a]				
	M. truncatula/G. versiforme (Days after inoculation)			*M. sativa* 'Kanza'/*G. versiforme* (Days after inoculation)	*M. sativa* MN NN-1008/*G. versiforme* (Days after inoculation)
Transcript	7	13	40	40	40
PAL	1.65 ± 0.5	1.7 ± 0.3	1.75 ± 0.25	2.0 ± 0.8	3.25 ± 0.25
CHS	2.25 ± 0.05	2.0 ± 0.05	1.8	1.75 ± 0.04	2.45 ± 0.25
IFR	1.1	0.6	0.4 ± 0.06	0.4 ± 0.02	1.9 ± 0.1

[a] Data are the mean values ± standard deviation for two to four replicates from pooled samples of five to twelve plants.

Flavonoid/isoflavonoid and transcript levels in a myc⁻ interaction.

Analysis of extracts from MN NN–1008/*G. versiforme* roots showed slight increases in daidzein (2-fold), FGM (1.6-fold), and MGM (2.4-fold) as compared to control MN NN–1008 root extracts (Figs. 3 and 6). Coumestrol and 4′,7-dihydroxyflavone were not present, whereas formononetin and medicarpin accumulated to 2.7 μg/g fresh weight and 1.3 μg/g fresh weight, respectively (Fig. 3). In general, this profile is most similar to the profiles of root extracts from *M. truncatula/G. versiforme* at 7 days after inoculation (Figs. 2B and 6B), and reflects the metabolite changes occurring in the very early stages of the interaction, during the period of external hyphal growth along the root surface and formation of the appressoria. Thus, it can be assumed that coumestrol and 4′,7-dihydroxyflavone accumulate during the internal growth of the fungus.

The levels of PAL, CHS, and IFR transcripts in MN NN–1008 control roots were comparable to those of *M. sativa* 'Kanza' and *M. truncatula* (Figs. 4 and 6). In contrast to the increases in PAL and CHS and decrease in IFR, transcripts observed in a complete mycorrhizal association (with either *M. truncatula* or *M. sativa* 'Kanza'), the levels of transcripts encoding PAL, CHS, and IFR in the myc⁻/*G. versiforme* interaction underwent 3.25-, 2.45-, and 1.9-fold increases, respectively, as compared with control roots of the same age (Fig. 6C; Table 1). Such coordinate increases in transcript levels, taken together with the increases in medicarpin and formononetin, suggest that a defense response may be occurring, albeit at a lower level than is usually observed in plant pathogen interactions.

DISCUSSION

We have chosen *M. truncatula* as a model plant in which to study the establishment and maintenance of a mycorrhizal symbiosis, as this species is a genetically tractable legume currently being developed as a model for the *Rhizobium*–legume symbiosis (Barker *et al.* 1990). We here report the first analysis of flavonoid/isoflavonoid compounds in roots of *M. truncatula* during the development of a mycorrhizal association, and demonstrate that the establishment of the symbiosis is accompanied by both qualitative and quantitative changes in many of the secondary metabolites of the root. The metabolite data are reflected by the profiles of transcripts encoding enzymes specific for phenylpropanoid, flavonoid, and phytoalexin biosynthesis. While coordinate regulation of these transcripts and phytoalexin biosynthesis has been previously observed in plant pathogen interactions, we here observe the suppression of transcripts encoding an enzyme specific for phytoalexin biosynthesis (and a subsequent de-

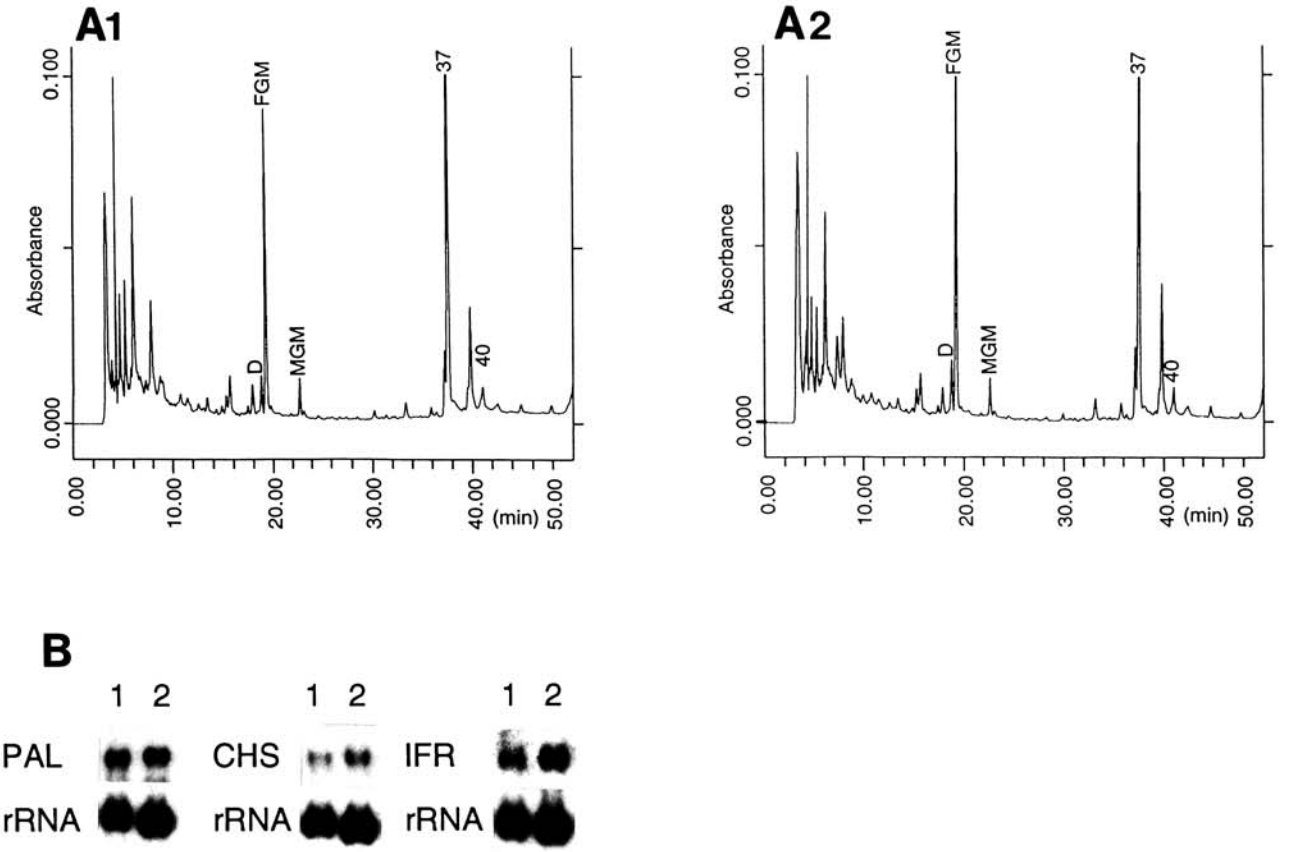

Fig. 5. The effects of growth in high phosphate conditions on the flavonoid profile and transcript levels in *M. truncatula* roots **A.** HPLC profiles of acetone extracts of *M. truncatula* roots grown for 40 days without additional phosphate (1) and with additional phosphate (2). **B.** Northern blot analysis of 2 μg of total RNA from *M. truncatula* roots grown for 40 days without additional phosphate (lane 1) and with additional phosphate (lane 2).

crease in phytoalexins), while an elevated level of transcripts encoding enzymes at earlier stages in the pathway is maintained.

Flavonoids/isoflavonoids observed in *M. truncatula/G. versiforme* interactions.

The most striking changes in the identified root metabolites include the overall accumulation of FGM, MGM, and daidzein and a transient increase in free medicarpin in the early stages of the interaction. Both MGM and FGM have been previously identified as major consitutive metabolites of *M. sativa* (alfalfa) and *Cicer arietinum* (chickpea) cell suspension cultures and roots. They accumulate in cell suspension cultures in response to fungal elicitors (Barz *et al.* 1989; L. Sumner, N. L. Paiva, and R. A. Dixon, unpublished results; Kessmann *et al.* 1990a, 1990b). The function of the conjugates is the subject of some speculation, but current data suggest that in alfalfa some MGM may be converted to medicarpin (the major phytoalexin) in the initial stages of a defense response (Dixon *et al.* 1992). In chickpea, under conditions when formation of early precursors of phytoalexin biosynthesis is inhibited, FGM can be converted to formononetin and channeled into phytoalexin biosynthesis (Barz *et al.* 1989). However, studies of defense responses in chickpea under normal physiological conditions suggest that generally FGM is not hydrolyzed and does not contribute to phytoalexin biosynthesis (Kessmann and Barz 1987).

The function of the increased levels of conjugates in mycorrhizae might be to provide a source of metabolites that could be rapidly mobilized under stress conditions such as pathogen attack. This source of precursors may be more important in the mycorrhizal state where the fungal symbiont may be consuming carbon precursors usually used for *de novo* synthesis of isoflavonoid phytoalexins. Alternatively, conjugation of medicarpin may inactivate or remove a potentially toxic metabolite. The toxicity of medicarpin to a number of fungal plant pathogens has previously been described (Higgins 1978); however, effects on VA fungi remain to be established.

Among the other flavonoids identified in *Medicago* mycorrhizal root extracts were daidzein and coumestrol, which have both been identified in mycorrhizal interactions in soybean (Morandi *et al.* 1984). The effects of daidzein on mycorrhizal fungi have not been reported; however, coumestrol is known to exhibit antinematode activity and may play a role in the reported resistance of mycorrhizal plants to nematode infestations (reviewed in Dehne 1982).

Emerging evidence suggests that certain flavonoids and isoflavonoids may play a role as signals in the mycorrhizal interaction. Formononetin was observed to

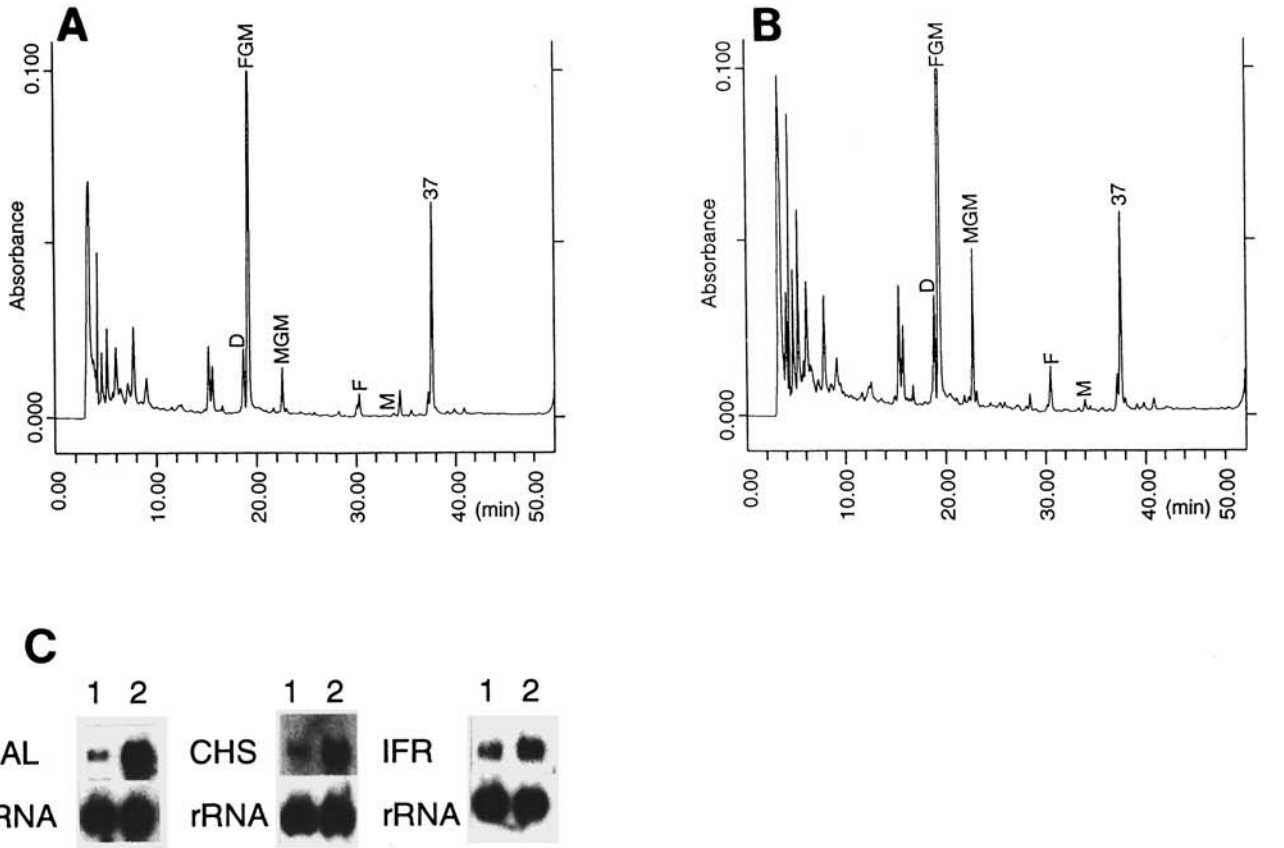

Fig. 6. Isoflavonoids and defense gene transcripts in roots of *M. sativa* MN NN-1008 (myc$^-$). (**A**), HPLC profiles of acetone extracts of *M. sativa* MN NN-1008 roots. (**B**), HPLC profiles of *M. sativa* MN NN-1008 roots after attempted colonization by *G. versiforme* (**C**), Northern blot analysis of 2 μg of total RNA from *M. sativa* MN NN-1008 during the interaction with *G. versiforme*. Lane 1: 2 μg of total RNA from uninoculated control roots (40 days). Lane 2: 2 μg of total RNA from roots after attempted colonization with *G. versiforme*; 40 days post inoculation.

stimulate the formation of interactions between clover and *Glomus* species (Siqueira *et al.* 1991) but to inhibit germination of spores from *G. etunicatum* (Tsai and Phillips 1991). 4′,7-dihydroxyflavone was demonstrated to stimulate VAM fungal spore germination *in vitro* (Tsai and Phillips 1991). Both of these compounds were detected in *Medicago* root extracts; but 4, 7-dihydroxyflavone was only present in extracts from mycorrhizal roots. Although the effects of various metabolites on aspects of fungal growth have been examined *in vitro*, it is difficult to extrapolate data from a single compound to the situation in whole roots where complex combinations of metabolites may have synergistic or antagonistic effects on each other.

In comparison to the complete mycorrhizal interaction, coumestrol and 4′,7-dihydroxyflavone did not accumulate in the interaction between the myc$^-$ *M. sativa* line and *G. versiforme*. It is possible that these compounds only accumulate after internal growth of the fungus. As the reason for the inability of MN NN-1008 to form a complete interaction is unknown, it is also conceivable that some compound required for signaling internal growth is missing in these lines. Coumestrol and 4′,7-dihydroxyflavone, along with other metabolites observed in the successful interactions, but not in myc$^-$ interactions, would be potential candidates for such a signal.

While many of the metabolites accumulate during the interaction, free medicarpin (the major phytoalexin in *M. sativa*) undergoes a transient increase in the early stages of the interaction and then decreases to very low levels by 40 days after colonization. As the control roots show a developmental decrease in the levels of free medicarpin, it is not possible, in the absence of further evidence, to attribute the decrease observed in the mycorrhizal roots to the fungal interaction. However, the levels of medicarpin in the successful interaction are much lower than in the myc$^-$ line at 40 days after colonization, suggesting that the decrease may not be solely a developmental effect.

In *M. sativa*, PAL and CHS are encoded by multigene families of at least four and seven genes, respectively (Gowri *et al.* 1991; Junghans *et al.* 1993), while IFR is encoded by a single gene (Paiva *et al.* 1991). *M. truncatula* contains multiple CHS genes (Junghans *et al.* 1993) and would be predicted to contain multiple PAL genes and a single IFR gene. The PAL and CHS probes used in this study hybridize to transcripts from all of the genes in their respective families. As multigene families frequently show differential regulation in response to different stimuli and to development (Wingender *et al.* 1989), it is possible that the increases in PAL and CHS transcripts observed here are specific for one member of the family. This could be determined with the use of gene specific probes.

The observed increases in the transcripts encoding PAL, CHS, and IFR in mycorrhizal roots are consistent with overall increased flavonoid biosynthesis. In contrast to the levels of PAL and CHS transcripts, which remain elevated throughout the interaction, the level of IFR transcripts decreases in later stages of the interaction to 2.5-fold below the level in the uninoculated control roots of the same age, suggesting that the established mycorrhizal association brings about a specific suppression of this transcript. This may be causally related to the decline in medicarpin levels observed in these roots. Analysis of IFR enzyme activity would further support this theory. Suppression of the defense response has been reported for certain interactions between plants and pathogenic fungi and suppressor molecules have been partially characterized (Doke *et al.* 1979; Barz *et al.* 1989; Yamada *et al.* 1989); however, the mechanisms by which these act is unknown.

Transient increases in free medicarpin coupled with increases in transcript levels for PAL, CHS, and IFR have been previously documented in *M. sativa* cell suspension cultures undergoing a defense response after treatment with fungal elicitors (Paiva *et al.* 1990). The patterns of medicarpin accumulation and changes in transcript levels observed here suggest that in the early stages of the interaction between *M. truncatula* and *G. versiforme* a defense response may have been initiated; however, it is clearly not sufficient to prevent further colonization and was not maintained. In addition, transcripts encoding a phytoalexin biosynthesis specific enzyme were suppressed below control levels. In the interaction between the myc$^-$ line and *G. versiforme* the levels of medicarpin and related transcripts would also suggest that a defense response is occurring. From these experiments it is not possible to determine whether the defense response is the cause or the effect of the failure of this myc$^-$ interaction.

The increases in phytoalexin levels and related transcripts observed here are several-fold smaller than reported during most documented defense responses (Dixon *et al.* 1992; Bell *et al.* 1986; Bhattacharyya and Ward 1986). This may be because the changes are the result of the direct interaction between fungal hyphae and a few plant cells, whereas measurements of metabolites and transcript levels are made in extracts of whole roots and include a larger number of noncolonized and, therefore, possibly nonresponding cells. Alternatively, the mycorrhizal fungi may simply avoid eliciting a significant defense response, a mechanism previously employed by certain plant pathogens (O'Connell *et al.* 1985; O'Connell and Bailey 1988).

The data presented here demonstrate significant changes in the root metabolite profiles of *M. truncatula* and *M. sativa* during the establishment of a mycorrhizal interaction, which provide a basis for further investigations into the role of flavonoids and isoflavonoids in this symbiosis. To further address the significance of changes in the levels of PAL, CHS, and IFR transcripts, we are currently using *in situ* hybridization to determine whether they are localized during the interaction. This approach enables a direct evaluation of colonized and noncolonized cells and is less compromised by the lack of synchrony of the mycorrhizal infection system.

MATERIALS AND METHODS

Stock cultures of VA mycorrhizal fungi.

Glomus versiforme (Karst) Berch. (kindly donated by

R. L. Peterson, University of Guelph, Canada) was multiplied and maintained on leek (*Allium porrum* L.) roots. To establish stock cultures, leek seeds were surface sterilized in 10% commercial bleach for 10 min, rinsed several times in sterile distilled water, and planted into sterile Turface (Plenchette *et al.* 1982) (A. H. Hummert Seed Co., St. Louis, MO). After 2 wk the seedlings were transplanted into turface containing *Glomus versiforme* spores and infected root pieces and stock cultures were established. Samples of roots from stock cultures were assessed microscopically for the establishment of mycorrhizal interactions. Uninoculated leek plants were maintained to provide a control inoculum. Both sets of leek stock plants were maintained in a growth chamber with 16-hr light, 8-hr dark cycles at 23° C (light) and 15° C (dark). Pots were watered with deionized water and fertilized once a week with 1/2× Hoagland solution without phosphate (Arnon and Hoagland 1940). Once a month the pots were watered with 1/2× Hoagland solution supplemented with 115 mg/L ammonium phosphate.

Plant growth and inoculation.

The plants used in this study included *Medicago truncatula* Gaertn 'Jemalong,' 2828 (Barker *et al.* 1990), *Medicago sativa* 'Kanza' and non-N_2-fixing *M. sativa* germplasm MN NN-1008 (kindly donated by D. K. Barnes, University of Minnesota). *M. sativa* MN NN-1008 has previously been demonstrated to be myc$^-$ (Bradbury *et al.* 1991). *Medicago sativa* 'Kanza' is one of the parental cultivars used to obtain MN NN-1008 (Barnes *et al.* 1988).

In all experiments, the seeds were surface-sterilized in 90% ethanol for 1 min, followed by 30% commercial bleach for 10 min, rinsed thoroughly in sterile distilled water, and planted into sterile Turface. The seedlings (15 days postgermination) were colonized with *G. versiforme* by transplanting them to pots containing a mixed inoculum of spores, hyphae and root pieces obtained from the leek stock cultures (Brundrett *et al.* 1984). Control seedlings were transplanted to pots containing control uninoculated leek root pieces.

The plants were maintained in a growth chamber with 16-hr light, 8-hr dark cycles at 23° C (light) and 15° C (dark). Pots were watered with deionized water and fertilized once a week with 1/2× Hoagland solution without phosphate (Arnon and Hoagland 1940). Phosphate was omitted as analysis of the Turface revealed that it contained low levels of phosphate (30 ppm P.). The growth response of *M. sativa* to mycorrhizae and phosphorous has been previously evaluated, and significant differences in growth between mycorrhizal and nonmycorrhizal plants were only observed when P levels were below 20 ppm (Lambert *et al.* 1980). In the present experiments visual observations suggested that there were no gross differences in the size or root mass of control and colonized plants.

In time course experiments, *M. truncatula* plants were harvested at 7, 13, and 40 days after inoculation. In experiments with *M. sativa* 'Kanza' and *M. sativa* MN NN-1008, the plants were harvested 40 days after inoculation. In experiments to assess the effects of phosphorous on the flavonoid/isoflavonoid content of the root, the phosphate-supplemented plants were watered once a week with 1/2× Hoagland solution containing 115 mg/L ammonium phosphate. Low phosphate plants were watered with 1/2× Hoagland solution without phosphate as previously described. Plants were harvested 40 days after inoculation.

Sample collection and assessment of colonization.

Each experimental sample contained five to 12 plants. These were removed from the Turface and washed thoroughly, and a random sample was removed for staining. As the amount of root obtained from the two earliest time points is low (from a subsequent experiment the root mass of 12 plants after 7 days of colonization was 1.01 g for both the colonized and control samples, and after 14 days of colonization was 2.12 and 1.84 g for the colonized and control samples, respectively) the roots were initially pooled, and 10–15% of the pooled sample was assessed for colonization. For the 40-day time point 10–15% of the individual plants were sampled for assessment of colonization.

Root samples were cut into segments approximately 1 cm long and stained with Chlorazol black E (Brundrett *et al.* 1984), and colonization was assessed microscopically (McGonigle *et al.* 1990; Bradbury *et al.* 1991). The length of root colonized in the 7- and 13-day samples was 30 and 70%, respectively. After 40 days, the individual plants showed between 50 and 90% colonization; however, only plants with colonization levels greater than 70% were selected for subsequent analyses. Root samples were pooled, crushed in liquid nitrogen to form a homogeneous mix of small root fragments, and stored in foil packages at –80° C for flavonoid, protein, and RNA analyses.

HPLC analysis.

Frozen root samples (0.1–0.25 g, which corresponds to 10–25% of early time point root samples) were ground to a fine powder in liquid N_2 and transferred to cold acetone (–20° C), and compounds were extracted overnight at 4° C (Edwards and Kessmann 1992). For each time point, samples of the pooled crushed roots were extracted and analyzed two to four times, depending on the amount of tissue available. Extracts were analyzed by HPLC linked to a diode array detector (Beckman Model 168) as described previously (Kessmann *et al.* 1990b). Metabolites were identified by comparing their retention times and UV spectra with those of known standards, and the identification was confirmed by coinjection with known standards. Estimates of the levels of metabolites (monitored at 287 nm) were made by comparing the peak heights with standards of known concentration. It has been confirmed that for these metabolites, the peak height provides a reliable estimate of the concentration.

RNA extractions and Northern blot analysis.

RNA was extracted from 0.1–0.2 g of root tissue using a modification of the procedure of Chromczynski and Sacchi (1987). A precipitation with 2 M LiCl was in-

cluded at the end of the preparation to ensure the complete removal of DNA.

RNA samples (1 μg) were separated by electrophoresis in formaldehyde gels (Sambrook *et al.* 1989) and blotted to Gene-Screen Plus as described in the Gene Screen (NEN, Hybridization Transfer Membrane) manual. ^{32}P-Labeled probes were prepared from cDNA inserts from pIFR alf1 (Paiva *et al.* 1991), alfalfa CHS2 (Junghans *et al.* 1993), pAPAL1 (Gowri *et al.* 1991), and pSR1-2B3 (18S rRNA) (Eckenrode *et al.* 1985) using standard methods (Sambrook *et al.* 1989). Hybridization conditions were as described in the Gene Screen manual.

The rRNA probe was used as a control for loading and transfer. Autoradiographs (short exposures) were scanned using JAVA, an automated video analysis system (Jandel) for quantitation of transcript levels. Transcript levels were normalized with respect to the rRNA to allow the calculation of changes in transcript levels shown in Table 1. All hybridization experiments were performed two to five times with samples from independent preparations of RNA and the average fold increases above control values were calculated.

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