Analysis of Parsley Arbuscular Endomycorrhiza: Infection Development and mRNA Levels of Defense-Related Genes

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Molecular aspects of the symbiosis between plants and arbuscular endomycorrhizal fungi have been investigated in parsley (*Petroselinum crispum*). Time course of infection with the inoculum *Glomus* sp. T6 was determined by estimating the total living or metabolically active fungal biomass. This revealed that phosphate nutrition and low light conditions negatively influenced plant-fungal interactions in different ways. Northern blot analyses of RNA from roots infected with the mycorrhizal fungus or from nonmycorrhizal roots of plants grown with or without phosphate fertilization were conducted using fresh weight as a basis for calibration. At the stage of highest symbiotic fungal activity, no dramatic changes were observable in the amount of plant mRNA encoding enzymes of the phenylpropanoid pathway and the methyl group cycle or the pathogenesis-related protein PR1. In contrast, two genes involved in cell wall modifications showed important differences. A transcript which codes for a hydroxyproline rich glycoprotein was produced in much higher amounts in mycorrhizal roots than in the uninfected controls. mRNA of a second gene encoding an anionic peroxidase showed strong accumulation after phosphate fertilization compared with the phosphate-deficient control and the mycorrhizal roots. Thin-layer chromatography revealed no large changes in the pattern of phenolic substances in root extracts, confirming the results concerning the phenylpropanoid pathway genes. New compounds could, however, be detected in the root exudates of phosphate-deficient plants.

Additional keyword: cell wall metabolism.

The arbuscular endomycorrhizal symbiosis is a widespread phenomenon found in most land plants (Tester *et al.* 1987), and because of its beneficial influence on the growth of crops (Plenchette *et al.* 1983), mostly due to a better mineral nutrition (Tinker 1975), it can be very useful for plant production (Gianinazzi *et al.* 1990; Bethlenfalvay 1992). In addition, mycorrhiza are a very attractive system for basic research on plant-microbe interactions because of the close cellular and physiological relationship between the two partners (reviewed in Gianinazzi-Pearson and Gianinazzi 1989). However, in contrast to pathogenic interactions, little is known about molecular events involved in infection and colonization of roots by arbuscular endomycorrhizal fungi (AEF). Of particular interest is the question of how the plant behaves during symbiotic processes in respect to mechanisms that are normally activated in interactions with pathogenic microorganisms. Defense responses seem to be low or only transiently activated in endomycorrhizas (Bonfante-Fasolo and Perotto 1990; Gianinazzi and Gianinazzi-Pearson 1990). In tobacco, the pathogenesis-related PRb1 gene was found to be weakly activated in mycorrhizal roots but not in controls, and the gene product could be localized in the interfacial matrix of the arbuscules, the central structure of the symbiosis (Gianinazzi-Pearson *et al.* 1992). Analysis of the level of transcription of other genes was conducted in bean mycorrhiza (Lambais and Mehdy 1993). In the symbiotic stage of the interaction, less mRNA was found encoding endochitinase, endoglucanase, and chalcone isomerase in mycorrhiza, while chalcone synthase (CHS) and phenylalanine ammonium lyase (PAL) gene activity remained constant. In contrast, in *Medicago truncatula,* an increase in PAL and CHS mRNA was observed after mycorrhization (Harrison and Dixon 1993), while expression of another gene encoding isoflavone reductase was enhanced at the beginning of root colonization and suppressed in later stages.

Parsley was chosen for the investigation presented here since it has been well investigated on a molecular level for its nonhost response to the pathogen *Phytophthora megasperma* f. sp. *glycinea*. In a parsley cell culture system, cDNAs have been isolated from transcripts which accumulate after *P. m.* f. sp. *glycinea* elicitor treatment (Somssich *et al.* 1986, 1989). It has been shown for two of these genes that their transcription is activated around *P. m.* f. sp. *glycinea* infection sites in parsley leaves during the hypersensitive response (Schmelzer *et al.* 1989). The possible function of some of these mRNAs has been elucidated (Kawallek *et al.* 1992; Kawallek 1991). They code for enzymes of the phenylpropanoid pathway or of the methyl group cycle and for cell wall modifying proteins. It therefore seemed interesting to use parsley for research of the expression of these factors in the symbiotic situation of mycorrhiza.

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We report here the establishment of parsley as a system for molecular investigations of arbuscular endomycorrhiza. A detailed infection analysis demonstrates the differential effects of light and phosphate nutrition on mycorrhization. In addition, it determines the stage of highest symbiotic fungal activity. For the molecular analysis, a calibration method is presented that takes into account the high infection level observed in parsley, and transcript patterns are investigated in the most active stage of the mycorrhizal development compared with two levels of phosphate nutrition. Results concerning genes involved in phenylpropanoid pathways are confirmed by analysis of the production of UV-fluorescing and UV-absorbing compounds.

RESULTS

Infection development.

The time course of colonization was measured in order to select a good mycorrhizal inoculum and to optimize conditions for infection and growth of parsley plants. The use of three different staining procedures in parallel (Tisserant *et al.* 1993) leads to a very detailed estimation of the level and activity of mycorrhization. Trypan blue stains all fungal material, while with an *in situ* enzyme assay for succinate dehydrogenase activity, living and dead hyphae can be distinguished. Finally, staining for fungal alkaline phosphatase (AP) activity differs between regions with low and high phosphate metabolism.

Parsley was inoculated with different fungi of the genus *Glomus* (*Glomus mosseae*, *Glomus* sp. D13, *Glomus* sp. T6, and *Glomus* sp. V49), and time courses of infection were followed. Comparing overall infection levels, the highest mycorrhization intensity was achieved with *Glomus* sp. T6. Figure 1 shows infection estimated by the three different staining procedures. While the degree of root colonization (M%) after trypan blue staining increased steadily over the whole experimental period, succinate dehydrogenase and AP activities reached a maximum after 4 wk and decreased during later stages of colonization.

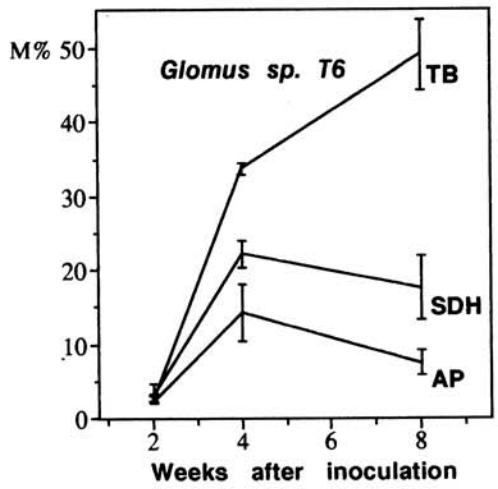

Fig. 1. Time course of infection. The intensity of mycorrhizal colonization (M%) of parsley root cortex 2, 4, and 8 wk after inoculation with *Glomus* sp. T6. Roots were stained with trypan blue (TB) or analyzed for fungal succinate dehydrogenase (SDH) and alkaline phosphatase (AP) activity. Data are the mean values of five replicates. Standard deviations are given as error bars.

In addition to the intensity of colonization (M%) shown in Figure 1, the frequency of infection (F%) and of arbuscule development (A%) were also estimated (Trouvelot *et al.* 1986). The importance of such a detailed infection analysis is illustrated by the differential influence of phosphate nutrition and light conditions on the mycorrhizal symbiosis (Fig. 2). It is well known that increasing phosphate and low light intensities inhibit mycorrhization (for references see Discussion). Looking more closely at the results with parsley, it can be seen that phosphate application influences the values of all three infection parameters, F%, M%, and A% (Fig. 2A). In contrast, under low light, the frequency of infection (F%) was hardly affected, but the intensities of colonization (M%) and arbuscule frequency (A%) were significantly reduced (Fig. 2B).

Molecular analysis.

All molecular analyses were carried out on plants inoculated with the isolate *Glomus* sp. T6. For the investigation of

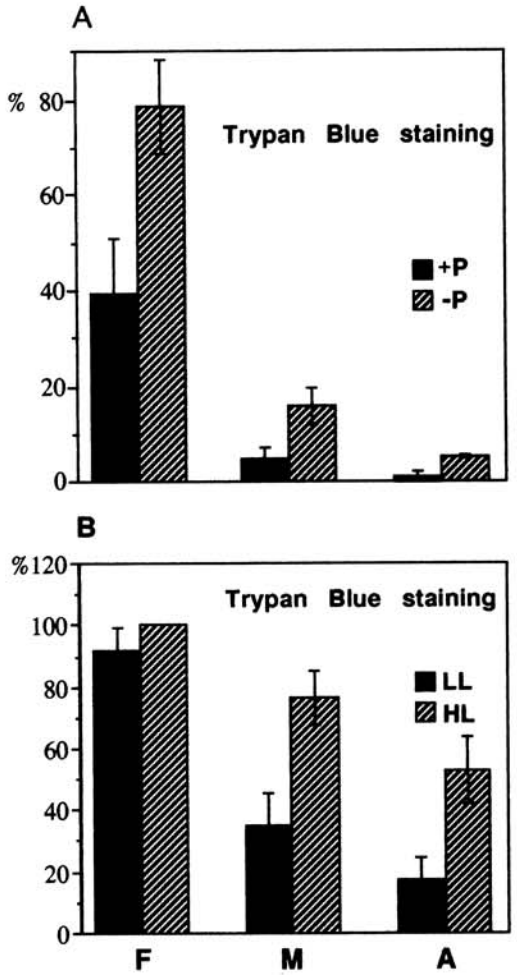

Fig. 2. Influence of light and phosphate on mycorrhization. Frequency of infection (F%), intensity of colonization (M%), and arbuscule frequency (A%) in mycorrhizal parsley roots, estimated 4 wk after inoculation with *Glomus* sp. T6. **A,** Plants were fertilized with normal Long Ashton solution (+P) or without the addition of NaH_2PO_4 (–P) and grown under low-light conditions. **B,** Plants were grown under low-light (LL = 190 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or high-light (HL = 420 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions without the addition of phosphate. Data are the mean values of three replicates. Standard deviations are given as error bars.

the symbiotic stage of mycorrhizal development, roots were harvested 4 wk after infection, when the AP activity had reached its maximum, because it has been previously reported that fungal AP activity is related to the symbiotic activity of the fungus (Gianinazzi-Pearson and Gianinazzi 1983; Smith and Gianinazzi-Pearson 1990; Tisserant *et al.* 1993). Parameters of infection were estimated after trypan blue staining. The frequency of infection (F) was 100%, the intensity of colonization (M) 74.3%, and the frequency of arbuscule development (A%) 53.2%. The mycorrhizal plants (–P,+AEF) showed at this time a clear growth effect producing nearly the same biomass (average shoot weight: 130 mg/plant) as fertilized plants (+P,–AEF; 145 mg/plant). The phosphate-deficient control plants (–P, –AEF) were much smaller (25 mg/plant). This material was used for the isolation of genomic DNA, RNA, and proteins, and for the extraction of phenolic compounds.

Calibration.

Investigations of the expression of plant genes in mycorrhiza and in control roots raise the problem of calibration. Comparing the same amount of RNA or proteins is biased by the presence of fungal compounds in the colonized sample. Furthermore, using a constitutively expressed gene as a control for hybridization was not suitable due to the differing morphology and physiology of mycorrhizal and control roots. This difference is reflected in the varying amounts of plant genomic DNA, total RNA, and proteins isolated from the three samples (Table 1). Total RNA and proteins were found in highest quantities in mycorrhiza (–P,+AEF), while in both controls (–P,–AEF and +P,–AEF), amounts were much less. The relative ratio of extractable RNA can be calculated as 4(–P,+AEF):3(+P,–AEF):2(–P,–AEF), and the relative amounts of protein as 4(–P, +AEF):2(+P,–AEF):1(–P,–AEF). A similar picture for crude protein extracts has been found in red clover, with a 2.6-fold higher yield of protein in mycorrhizas than in uninfected roots (Arines *et al.* 1993). In contrast, the amount of plant genomic DNA, measured by Southern blot analyses, was similarly high in roots of phosphate-sufficient plants, either fertilized (+P,–AEF) or mycorrhizal (–P,+AEF), but five times less in the phosphate-deficient control (–P,–AEF).

These observations led to the conclusion that the usual calibration procedures used for Northern blot experiments may bias the investigations of mRNA levels in mycorrhiza. Moreover, reference to cell number measured by the amount of plant genomic DNA did not seem reliable. Therefore, analyses in the following experiments were calibrated on a fresh weight basis. Error during sampling and extraction was minimized by taking the average of four replicate experiments. The standard deviation was found to be less than 10% (Table 1).

Table 1. Amounts (µg) of plant DNA, total RNA, and protein extracted from 1 g fresh weight of roots[a]

Substance	–P –AEF[b]	+P –AEF	–P +AEF
Plant DNA[c]	10.6 ± 0.9	62.4 ± 3.1	49.3 ± 2.3
Total RNA[d]	122 ± 4	169 ± 6	247 ± 8
Protein[e]	600 ± 23	1,280 ± 32	2,320 ± 53

[a] Given as mean values ± standard deviation of two (DNA) or four (RNA and protein) extractions from one infection.
[b] P = phosphate; AEF = arbuscular endomycorrhizal fungi.
[c] Experiment calculated by Southern blot hybridization (Materials and Methods).
[d] Calculated by measuring OD_{260}.
[e] Calculated according to Bradford (1976).

Northern blot experiments.

Initial Northern blot analysis using total RNA showed that the relative abundance of the investigated transcripts was so small that their position in the blots was biased by the mass of ribosomal RNA (data not shown). Poly(A)$^+$ RNA was therefore purified. The Northern blots were hybridized with probes for genes whose expression is activated in parsley cell cultures by *P. m.* f. sp. *glycinea* elicitor (Somssich *et al.* 1989), with the exception of the light-inducible chalcone synthase gene (CHS; Chappell and Hahlbrock 1984) and the presumably constitutively expressed genes encoding polyubiquitin (Ubi; Kawallek *et al.* 1993) or glycerin aldehyde phosphate

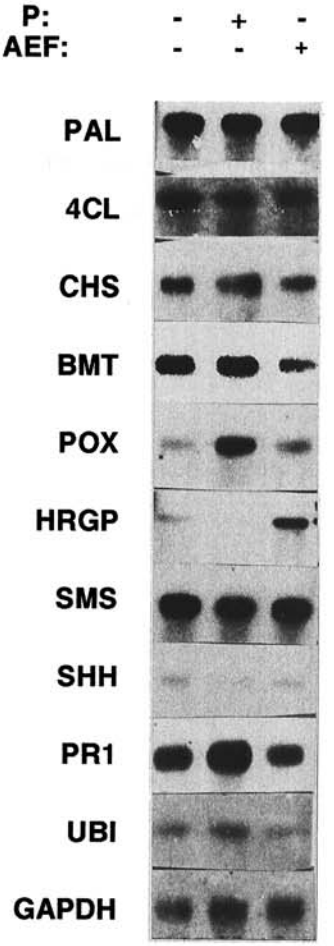

Fig. 3. Northern blots. Poly(A)$^+$ RNA was isolated from 20 µg (–P, –AEF), 30 µg (+P,–AEF), or 40 µg (–P, +AEF) total RNA and analyzed on Northern blots. ^{32}P-labeled DNA inserts of cDNA clones previously isolated by Somssich *et al.* (1986, 1989) served as probes. Abbreviations used for the corresponding genes are explained in the results. Plants were fertilized with (+P) or without (–P) phosphate, inoculated with Glomus sp. T6 (+AEF), or not inoculated (–AEF).

dehydrogenase (GAPDH; Martin *et al.* 1989). The products of the other genes represent three enzymes of phenylpropanoid biosynthesis—phenylalanine ammonium-lyase (PAL), 4-coumarate:CoA ligase (4CL) (Kuhn *et al.* 1984), and bergaptol *O*-methyltransferase (BMT; Hauffe *et al.* 1986)—or of the methyl group cycle—*S*-adenosyl-L-methionine synthetase (SMS) and *S*-adenosyl-L-homocysteine hydrolase (SHS) (Kawallek *et al.* 1992). Others are involved in cell wall modifications as genes coding for a hydroxyproline-rich glycoprotein (HRGP) or for an anionic peroxidase (POX) (Kawallek 1991). One elicitor-responsive gene of unknown function (PR1; Somssich *et al.* 1988) was also included in this analysis.

Figure 3 shows the autoradiographs of the Northern blots that were obtained on an equivalent fresh weight basis for the reasons explained above. The hybridization intensities were equivalent whether total or poly(A)$^+$ RNA was used. Quantification of the results by image analysis of these blots is shown in Table 2. Values in brackets have been corrected for equal amounts of RNA loading based on a factor calculated from the results given in Table 1.

Gene expression concerning the general phenylpropanoid pathway PAL and 4CL as well as the flavonoid pathway CHS did not seem to be significantly influenced by any treatment. Only the signal intensity with the probe for the BMT gene was slightly reduced in the mycorrhizal sample (–P,+AEF). The expression of the two genes involved in cell wall metabolism showed a strong variation. While HRGP mRNA accumulated to a very high level in mycorrhiza (–P,+AEF) and decreased after phosphate fertilization (+P,–AEF), the POX gene was clearly induced in the phosphate-fertilized control (+P,–AEF). A similar pattern as with POX was seen with the PR1 probe, although activation by phosphate nutrition was much lower. The inverse behavior could be detected for the SHH gene whose transcription seemed to be repressed by phosphate fertilization (+P,–AEF). The SMS and the GAPDH genes were not significantly influenced, while the Ubi gene appeared to be most strongly transcribed in the phosphate fertilized controls (+P,–AEF) and least in mycorrhizal roots (–P,+AEF).

Results based on equal RNA loading (Table 2; values in brackets) indicate that expression of most of the investigated genes are reduced slightly after phosphate fertilization (+P, –AEF) and even more in mycorrhizal roots (–P,+AEF). For SHH, the greatest reduction was induced by phosphate (+P, –AEF). For PR1 and Ubi, the effects of phosphate fertilization (+P,–AEF) on the amounts of transcripts are attenuated, while the reduction in mRNA accumulation due to mycorrhization (–P,+AEF) is amplified. Only results for POX and HRGP were relatively unchanged by the mode of calibration.

Table 2. Relative changes in the mRNA levels in phosphate-fertilized controls (+P,–AEF) and mycorrhiza (–P,+AEF)[a]

Gene	Change (-fold) over phosphate-deficient control (–P,–AEF)	
	+P,–AEF	–P,+AEF
PAL	0.9 (0.65)[b]	0.88 (0.43)
4CL	0.86 (0.62)	0.85 (0.42)
CHS	0.82 (0.59)	0.82 (0.4)
BMT	0.99 (0.71)	0.59 (0.29)
POX	3.62 (2.61)	1.34 (0.66)
HRGP	0.55 (0.36)	6.64 (3.27)
SMS	0.92 (0.67)	0.99 (0.49)
SHH	0.45 (0.34)	0.82 (0.4)
PR1	1.78 (1.29)	0.88 (0.43)
Ubi	1.97 (1.42)	0.7 (0.33)
GAPDH	1.05 (0.76)	1.12 (0.55)

[a] AEF = arbuscular endomycorrhizal fungi.

[b] Values in parentheses are corrected for equal amounts of RNA loading based on a factor calculated from the results in Table 1.

Phenolic compounds.

In order to look for correlations between the transcriptional levels of the phenylpropanoid pathway genes and the corresponding products, methanolic extracts of parsley roots were purified and analyzed by thin-layer chromatography. This was repeated six times under different conditions, because quantities could vary between extracts from the same sample. The results indicated that there was no dramatic activation or repression in synthesis of a certain group of phenolic compounds, such as that observed for furanocoumarins after elicitor treatment or for flavonoids after light induction in parsley cell cultures (Dangl *et al.* 1987). This was in agreement with the results of the transcriptional analysis calibrated on a fresh weight basis.

Minor changes did occur, however, in phenolic components, and among these, one is particularly obvious in Figure 4A. Roots of plants grown under phosphate stress (–P, –AEF) produced compounds (indicated with arrows) that are absent from the phosphate-fertilized control (+P,–AEF) and from mycorrhiza (–P,+AEF). This difference was also detected in root exudates where one compound was detected in the phosphate-deficient control (–P,–AEF) that was clearly absent from the other two samples (Fig. 4B).

DISCUSSION

The use of three different staining methods (Tisserant *et al.* 1993) combined with the calculation method of Trouvelot *et al.* (1986) led to a suitable estimation of the level of mycorrhization in parsley plants. With these methods, optimal sampling time of plant material could be determined for the molecular experiments and interesting differences observed concerning environmental influences. With the various inocula, parsley mycorrhiza showed a typical time course of colonization observed before in other plants (Tisserant *et al.* 1993), but using the inoculum *Glomus* sp. T6 (Grunewaldt-Stöcker and Dehne 1989) under optimal conditions, the highest values for mycorrhizal intensity (M%) and arbuscule frequency (A%) were obtained. In addition, mycorrhizal plants showed a typical growth response to infection, indicating the success of the symbiosis. These observations represent the first report of arbuscular mycorrhizal infection and growth effects in parsley, and underline the interest of this plant for studies of arbuscular endomycorrhiza.

Both light intensity and phosphate levels were critical factors affecting mycorrhiza development in parsley. This has also been observed in other plants (reviewed in Gerdemann 1968; Gianinazzi-Pearson and Diem 1982). Concerning light, the effect seems to be dependent on the plant species used for the analysis. In parsley, mycorrhiza development shows a clear response to light, but only the levels of colonization and arbuscule frequency were reduced. One explanation could be

that fewer assimilates are produced. This would reduce the amount of carbohydrates in the root and is probably the reason for the limited spread of the fungus, as already pointed out a long time ago for ecto- (Björkmann 1942) and endomycorrhiza (Peuss 1958).

The negative influence of phosphate on mycorrhization has also been well known for a long time (Mosse 1973; Sanders 1975). Several investigators have indicated that the number of entry points is mainly affected (Jasper *et al.* 1979; Schwab *et al.* 1983; Thomson *et al.* 1986), but inhibition of the intraradical spread of the fungus has also been suggested as a major reason (Amijee *et al.* 1989; Braunberger *et al.* 1991). In the study presented here, phosphate nutrition seemed to influence all the critical stages of infection in a similar manner in parsley.

The fact that the frequency of infection, which partly reflects the extraradical spread of the fungus from one root to another, is influenced by phosphate levels could be linked to the production of certain signal molecules for the fungus in roots under phosphate stress. It has been shown that root exudates of phosphate-deficient white clover plants were much more active in enhancing hyphal elongation from spores of arbuscular endomycorrhizal fungi than exudates of fertilized plants (Elias and Safir 1987). In root exudates of phosphate-stressed parsley, one particular substance was specifically present. This compound will be analyzed in further investigations for its structure and influence on spore germination and hyphal growth in order to determine its eventual relevance to host-fungal interactions in arbuscular endomycorrhiza.

Alternatively, the lower mycorrhizal colonization in phosphate-sufficient plants may be explained by the induction of some kind of defense response. In soybean, for example, an increase in phytoalexin compounds after phosphate fertilization was shown in roots (Morandi and Gianinazzi-Pearson 1986). The molecular analyses in our study revealed that in parsley, genes encoding an anionic peroxidase and PR1 showed an enhanced transcription under high phosphate conditions, and this was independent of the mode of calibration for the anionic peroxidase. Whether the higher expression of these two genes contributes to an induced resistance to the mycorrhizal fungi after phosphate fertilization is difficult to test in parsley, since no corresponding mutants yet exist for this system. However, in potato, where a similar increased peroxidase enzymatic activity in root extracts of phosphate-fertilized plants was found (McArthur and Knowles 1992), such questions could be answered.

Previous histological and cytological investigations indicate that arbuscular endomycorrhiza and uninfected plant roots are quite distinct tissues. A very detailed analysis concerning the overall morphology and the mitotic index was published by Berta *et al.* (1993), the results of which suggest that important modifications occur in plant tissues so that gene expression in mycorrhiza might be difficult to compare with that in uninfected roots, even without taking into account the presence of the fungus. Probes to ribosomal RNA could not be used, because differences in size between fungal and plant rRNA reported by Judelson and Michelmore (1990) were not detected for rRNA from AMF spores and parsley roots (data not shown). For this reason, a calibration method had to be used for the molecular investigations that would be independent of morphology and metabolic activity. The use of fresh weight fulfilled the criteria and had an additional advantage in that different kinds of molecules (DNA, RNA, proteins, etc.) could be measured on the basis of one reference value. The mistake that could have been introduced by variations in sampling and extraction was found to be too small to bias the results.

Calibration on a fresh weight basis has been used before, e.g., for the study of carbohydrate profiles (Amijee *et al.* 1993) or phenolic compounds (Morandi *et al.* 1984; Wyss *et*

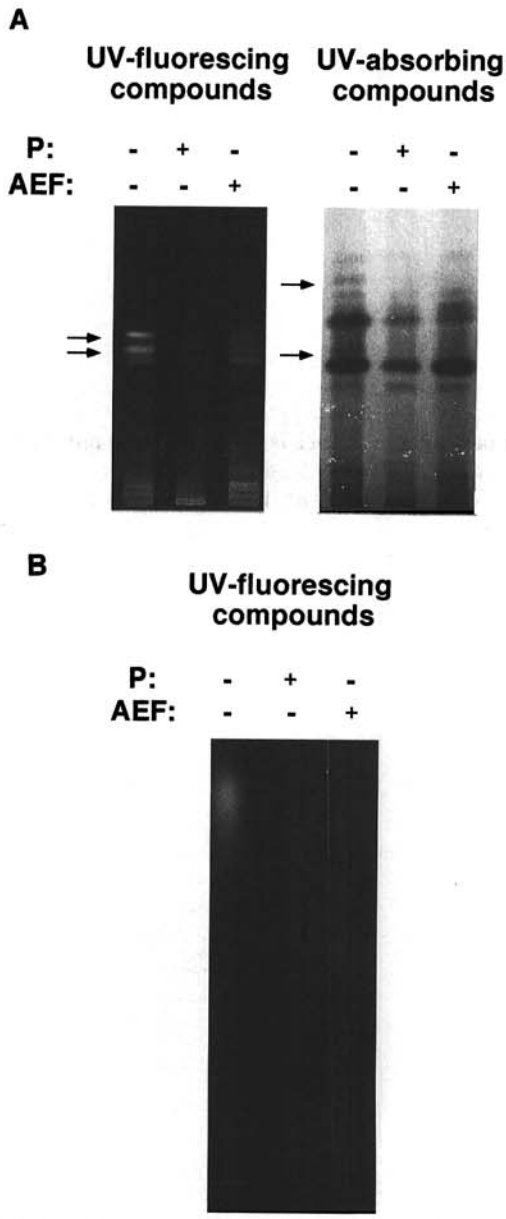

Fig. 4. Thin-layer chromatography. **A,** Methanol extracts of total roots or **B,** CH_2Cl_2 extracts of root exudates obtained from different treatments and analyzed by thin-layer chromatography. Analysis was conducted on silica gel (A), with a fluorescent indicator at two different wave lengths: 366 nm for UV fluorescence or 245 nm for UV absorbance. Exudates (B) were investigated on cellulose at 366 nm. The presence of additional compounds in the phosphate-deficient sample in A is indicated by arrows (±P,±AEF; see Figure 3 for explanation).

al. 1991) in mycorrhiza. In the present investigations, it led to reliable results, illustrated by comparison of the results of the Northern blot experiments and the analysis of the phenolic compounds from parsley roots. The unmodified transcript level of the three phenylpropanoid pathway genes, PAL, 4CL, and CHS, is in good agreement with the absence of any major quantitative change in phenolic compounds in any treatment. In contrast, if the amount of RNA is used as a reference, all these transcripts show a reduction in mycorrhiza, which does not correspond to the analysis of the phenolic compounds. Lack of changes in cell wall–bound phenols with mycorrhiza formation has been shown previously in leek and in ginkgo (Codignola *et al.* 1989). Similarly, no differences in signal intensity have been observed in Western blots with a PAL and a CHS antiserum between mycorrhizal and nonmycorrhizal roots of parsley (data not shown). Further evidence that fresh weight is more suitable for calibration comes from the analysis of GAPDH expression; the level of transcripts encoding GAPDH expressed on a fresh weight basis does not seem to be modified, whereas loading of equal amounts of RNA would lead to a reduced amount of GAPDH mRNA in mycorrhiza. This is relatively unlikely, because carbohydrates are exchanged between the two partners of the symbiosis, and therefore, enzymes involved in this metabolism should not be less expressed.

In contrast to the other genes of the phenylpropanoid pathway, BMT transcripts were found in lower amounts in mycorrhizal parsley roots. BMT is involved in the production of the furanocoumarin phytoalexins following elicitor treatment of parsley cells (Hauffe *et al.* 1986) or fungal infection of parsley leaves (Jahnen and Hahlbrock 1988; Schmelzer *et al.* 1989). The lower transcript level observed here may reflect repression of the production of fungitoxic phytoalexins in mycorrhiza during fully established and active symbiosis. With respect to this hypothesis, results published up to now differ. Whereas mycorrhizal infection seems to induce the production of phytoalexins in *Vicia faba* (Kape *et al.* 1992) and in soybean (Morandi *et al.* 1984), Wyss *et al.* (1991) reported no phytoalexin response in another soybean cultivar during the colonization with a symbiotic fungus. Similar to parsley, in *Medicago truncatula*, transcripts encoding the phytoalexin-specific enzyme isoflavone reductase were suppressed in late stages of mycorrhiza development (Harrison and Dixon 1993). Also in *Medicago sativa*, it was deduced from enzyme data that an initial defense response is later suppressed (Volpin *et al.* 1994). Consequently, more investigations are necessary with different plants to clarify the role of fungitoxic compounds in arbuscular endomycorrhiza.

Genes that are involved in cell wall synthesis and modification represent those that are most influenced by phosphate nutrition or mycorrhization. The significant accumulation of HRGP-encoding mRNA in parsley during the symbiosis independent of the mode of calibration is in agreement with the detection of an HRGP in the arbuscule matrix between the fungus and plant in leek cells (Bonfante-Fasolo *et al.* 1991). However, preliminary *in situ* hybridization indicates that in parsley the transcript accumulates not in arbuscule cells but in the central cylinder and tip of mycorrhizal roots. This discrepancy may be due to the presence of several different HRGP genes in most plants, while the probe used here seems to detect only one single copy sequence in the parsley genome (Kawallek 1991). Further investigations, including immunocytochemistry with antisera to various HRGP, should clarify this point.

Expression of the elicitor-responsive gene SHH involved in the activated methyl group cycle is slightly enhanced in the phosphate-deficient control and in mycorrhizal roots of parsley. Methylation is involved in so many processes concerning different metabolic pathways that a discussion of the significance of this observation is virtually impossible. Polyubiquitin also has many different roles. However, the fact that the corresponding gene is expressed at a lower level in mycorrhiza could be related to the reduced mitotic activity of infected root systems (Berta *et al.* 1993), since ubiquitin is involved in the degradation of certain proteins during the cell cycle (Jentsch *et al.* 1990). The Ubi gene has been used to calibrate Northern blots in the analysis of the accumulation of different transcripts after elicitor or wound induction in parsley (Dangl *et al.* 1987; Lois and Hahlbrock 1992). The results presented here show that this is not possible for the investigation of the transcription in arbuscular endomycorrhizal parsley roots.

In conclusion, the present results show that most of the investigated genes, which are normally strongly induced in parsley by elicitor treatment or during nonhost responses in leaves, were not, at the time of analysis, more highly expressed in arbuscular endomycorrhiza than in control roots, even though the tissue was highly infected. This suggests that during symbiotic interactions the mycorrhizal fungi may have the ability to circumvent the plant's response to attack by microorganisms at the transcriptional level. This does not appear to result from symbiotic fungi lacking eliciting compounds, since the activation of defense responses has been described in certain pea mutants unable to develop mycorrhiza (Gianinazzi-Pearson *et al.* 1991; Gollotte *et al.* 1993). It seems more likely that active repression of defense gene transcription occurs. The existence of mycorrhiza-resistant pea mutants, which responded to the fungi as if attacked by a pathogen, suggests the presence in normal hosts of plant symbiotic genes that are antagonistic to defense-related genes, controlling their activity during mycorrhizal development (Gollotte *et al.* 1993). In parsley, the activation of elicitor-induced genes has been studied in detail, including the architecture of promoters and the involvement of DNA-binding proteins and regulatory genes (Lois *et al.* 1989; van de Löcht *et al.* 1990; Meier *et al.* 1991; Douglas *et al.* 1991; Da Costa e Silva *et al.* 1993). Parsley may therefore be a suitable system for investigating the suppression of defense responses in arbuscular endomycorrhiza at the molecular level.

MATERIALS AND METHODS

Fungal strain.

The different *Glomus* sp. inocula were provided by H. von Alten (University of Hannover). They consisted of spores and hyphae in an expanded clay carrier material (Lecaton; Leca Deutschland GmbH, Halstenbeck, Germany; Grunewaldt-Stöcker and Dehne 1989). *Glomus mosseae* (Nicol. & Gerd.) spores were obtained from the Institute National de la Recherche Agronomique (INRA), Dijon (isolate LPA 5).

Plant material, inoculation and growth.

Parsley seeds (*Petroselinum crispum* cv. Hamburger Schnitt) were surface-sterilized, germinated, and grown in multipot plates under conditions previously described (Knogge *et al.* 1987). After they had reached the one-leaf stage, single plants were transferred to pots containing expanded clay as substratum and grown under constant conditions: 75% relative humidity, 25°C, 16-hr day (190 μmol $m^{-2} \cdot s^{-1}$ with neon lights or 420 μmol $m^{-2} \cdot s^{-1}$ with sodium vapor lights). For mycorrhization, the substratum was mixed 1:10 with the inoculum. Fertilization was done weekly with 5-ml Long Ashton solution (Hewitt 1966) containing 66.7 nM phosphate per 100 ml substratum. For the mycorrhizal plants and the –P controls, the fertilizer was supplied without NaH_2PO_4.

Staining.

At different times after inoculation, roots of five plants were pooled and stained with trypan blue or for fungal succinate dehydrogenase and alkaline phosphatase activities (Tisserant *et al.* 1993). Two plants in the –P and +P controls were checked. Mycorrhization was estimated as previously described (Trouvelot *et al.* 1986). Briefly, stained roots were cut into 1-cm pieces and mounted on slides. The frequency of infection (F%) was calculated as the relative number of infected root pieces, the colonization intensity (M%) as the proportion of colonized to total root length, and arbuscule development (A%) as frequency of arbuscules per total root length.

Determination of plant genomic DNA content.

Genomic DNA was isolated from roots with the miniprep method of Dellaporta *et al.* (1983), and an amount corresponding to 100 mg fresh weight was digested with *Eco*RI and analyzed by Southern blot (Kawallek *et al.* 1992). For the comparison of plant genomic DNA content in the three samples, the signal intensity was estimated with an image analysis video system (SAMBA) after hybridization to a CHS and a PAL cDNA probe (Kreuzaler *et al.* 1983; Kuhn *et al.* 1984) labeled with ^{32}P (Feinberg and Vogelstein 1984). Ten micrograms of genomic DNA isolated from cultured parsley cells (Murray and Thompson 1980) was used as a standard.

Determination of protein content.

Proteins were extracted by grinding 100 mg of root material in an Eppendorf tube in 200 μl of TMK buffer (10 mM Tris/Cl pH 7.5; 10 mM $MgCl_2$; 20 mM KCl; 0.25 mM EDTA; 2 mM 2-mercaptoethanol). Protein content was measured following the method of Bradford (1976) calibrated using bovine serum albumin.

Northern blot.

Total RNA was isolated following the method of Logemann *et al.* (1987) but with a modified extraction buffer (50 mM Tris/Cl, pH 9; 150 mM NaCl; 5 mM EDTA; 5% SDS). Poly(A)RNA was purified with oligo(dT)-Dynabeads (Dynal GmbH, Hamburg, Germany) using total RNA from about 170 mg fresh weight of each sample and was directly subjected to the Northern blot analysis. Running of the gel, transfer to Hybond N (Amersham, Arlington Heights, IL), and hybridization with cDNA probes was done as described by Kawallek *et al.* (1992). All experiments shown were done with two membranes. Before reprobing, they were stripped 2 × 30 min in 0.1% SDS at 80°C. Membrane 1 was hybridized in the following order with UBI, HRGP, BMT CHS and SMS, membrane 2 with SHH, GAPDH, POX, 4CL, and PR1. Finally, both membranes were probed with PAL. The autoradiographs were scanned using SAMBA, an image analysis video system.

Extraction and analysis of phenolic compounds.

Extracts were prepared and analyzed as described by Dangl *et al.* (1987). One gram of root material (fresh weight) was crushed in 4 ml of 80% MeOH with an ultraturax (Jahnke und Kunkel, Staufen, Germany). After removing the cell debris by centrifugation (10 min, 20,000 × *g*), the supernatant was reduced under vacuum, hydrolyzed for 1 hr in 1 N HCl at 95°C, reduced again, and dissolved in MeOH. The analysis was conducted on precoated silica gel 60 plates with a fluorescence indicator (Merck, Darmstadt, Germany) using toluene/ethylformiate/formic acid (5:4:1; v/v) as the solvent system.

Analysis of root exudates.

Water (50 ml) was run through four pots per treatment, pooled, and extracted with the same volume of CH_2Cl_2. The organic phase was reduced under vacuum; the pellets were redissolved in MeOH and analyzed on precoated cellulose plates (Merck) with water-saturated chloroform/acetic acid (3:2; v/v) as the solvent.

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