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Changes in polypeptide patterns in tobacco roots colonized by two *Glomus* species

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Abstract. Changes in gene expression were studied during the establishment of arbuscular mycorrhizal symbiosis in tobacco roots from an amphidiploid hybrid *Nicotiana glutinosa* × *N. debneyi.* Polypeptide patterns from control roots and from roots infected by *Glomus mosseae* or *G. intraradices* were resolved by two-dimensional polyacrylamide gel electrophoresis and followed in a time-course analysis. Arbuscular mycorrhizal infection led to significant modifications in polypeptide patterns with: (a) decreased amounts of some polypeptides, (b) increased accumulation of others, and (c) appearance of newly-induced polypeptides. Comparisons made during infection development by the two *Glomus* species demonstrated that protein modifications changed in relation to the mycorrhizal state of the tobacco roots.

Key words: *Nicotiana* – *Glomus* species – arbuscular mycorrhiza – gene expression – specific polypeptides

Introduction

The formation and function of arbuscular mycorrhiza require complex morphogenetic, biochemical, physiological and molecular changes in both fungi and roots. Although knowledge concerning the molecular basis of mycorrhizal associations is still in its infancy (Gianinazzi 1991; Martin and Hilbert 1991; Bonfante-Fasolo and Perotto 1992), there is some evidence for the production of new, specific polypeptides or proteins in response to ectomycorrhiza (Hilbert and Martin 1988; Martin and Hilbert 1991; Hilbert et al. 1991) and arbuscular mycorrhiza ((Dumas et al. 1989; 1990; Schellenbaum et al. 1992; Garcia-Garrido et al. 1993) formation. Some mycorrhiza-specific polypeptides have also been reported to immunologically cross-react with antibodies against nodule-specific proteins (nodulins) (Wyss et al. 1990).

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However, there are difficulties in identifying the true origin of the observed modifications in gel electrophoresis protein patterns due to the fact that arbuscular mycorrhizal fungi are obligate symbionts and, therefore, cannot be grown axenically. Furthermore, the resolution obtained by one-dimensional gel electrophoresis (1D-PAGE) is not sufficient to detect polypeptides or proteins that are probably produced transiently, and/or in small amounts, during the establishment of the arbuscular mycorrhizal symbiosis.

The aim of the present work was to use 2D-PAGE to study the modifications in polypeptide patterns linked to endomycorrhizal infection of tobacco roots. This technique has been successfully used to identify quantitative and qualitative changes occurring in soluble polypeptides of salt-stressed tissues (Hurkman and Tanaka 1987), pathogen-infected plant tissues (Hadwiger and Wagoner 1983; Sang-Gu and Joo-Yeon 1992) and also symbiotic tissues in roots (Hilbert and Martin 1988; Krause and Broughton 1992). We have investigated modifications of polypeptide patterns in root extracts from an amphidiploid hybrid *Nicotiana glutinosa* × *N. debneyi* obtained in our laboratory (Ahl and Gianinazzi 1982; Ahl et al. 1983) during arbuscular mycorrhiza formation by two fungi, *Glomus mosseae* and *G. intraradices.* The observed changes included both repression and induction of protein synthesis. Furthermore, differences also occurred in the times of appearance or disappearance of some polypeptides depending on the fungal species, providing further evidence for the existence of polypeptides specific to the symbiotic state in mycorrhiza.

Material and methods

Plants and growth conditions

About 300 surface-sterilized seeds of the amphidiploid hybrid of *N. glutinosa* × *N. debneyi* were sown in 9-l plastic pots containing a γ-irradiated soil (clay loam, pH 7.6, 26 ppm Olsen phosphorus). Plants were inoculated with either *G. mosseae* (Nicol. & Gerd.) (isolate LPA 5) or *G. intraradices* (Schenck & Smith) (iso-

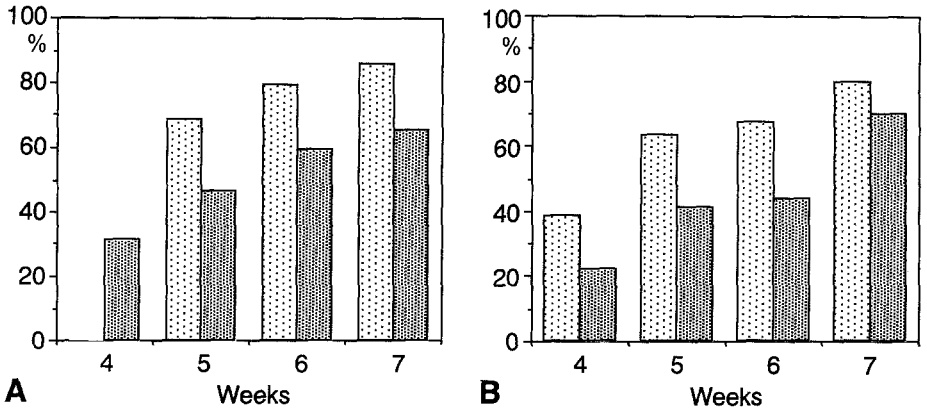


Fig. 1. Percent of colonized cortical tissue (M%; *light dotted column*) and intensity of arbuscule development (A%; *dark dotted column*) in roots from the amphidiploid hybrid *Nicotiana glutinosa* × *N. debneyi* colonized by **A** *Glomus mosseae* and **B** *G. intraradices*

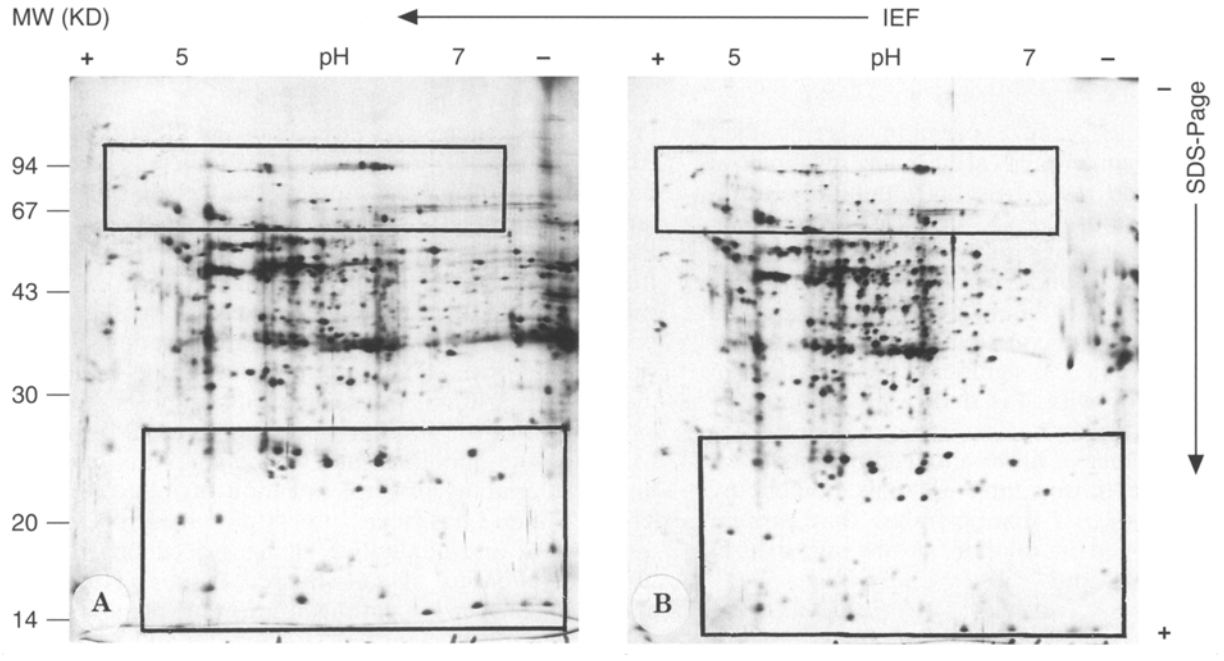


Fig. 2. Silver-stained two-dimensional gels of extracts of **A** uninoculated roots from the *N. glutinosa* × *N. debneyi* hybrid and **B** roots colonized by *G. mosseae* after 4 weeks. First dimension: isoelectric focussing in a pH gradient of 4.5–7.5; second dimension: 12% sodium dodecylsulphate polyacrylamide gel. Aliquots of 100 μg protein of *G. mosseae*-infected roots and 60–90 μg (corresponding to the same fresh weight of roots) of uninoculated control roots were loaded onto the gels. Upper and lower *boxes* show the selected parts of the 2D-PAGE presented in Figs. 3 and 4

late LPA 8) using root inoculum from a stock culture of *Allium porrum* L. cv. Verina, or grown in disinfected soil as nonmycorrhizal control plants. Plants were grown in a controlled environment room (22° C, 60–77% relative humidity, 16-h photoperiod at 220 μmol m^{-2} s^{-1}). They were watered weekly with Long Ashton solution without phosphorus, and daily with deionized water.

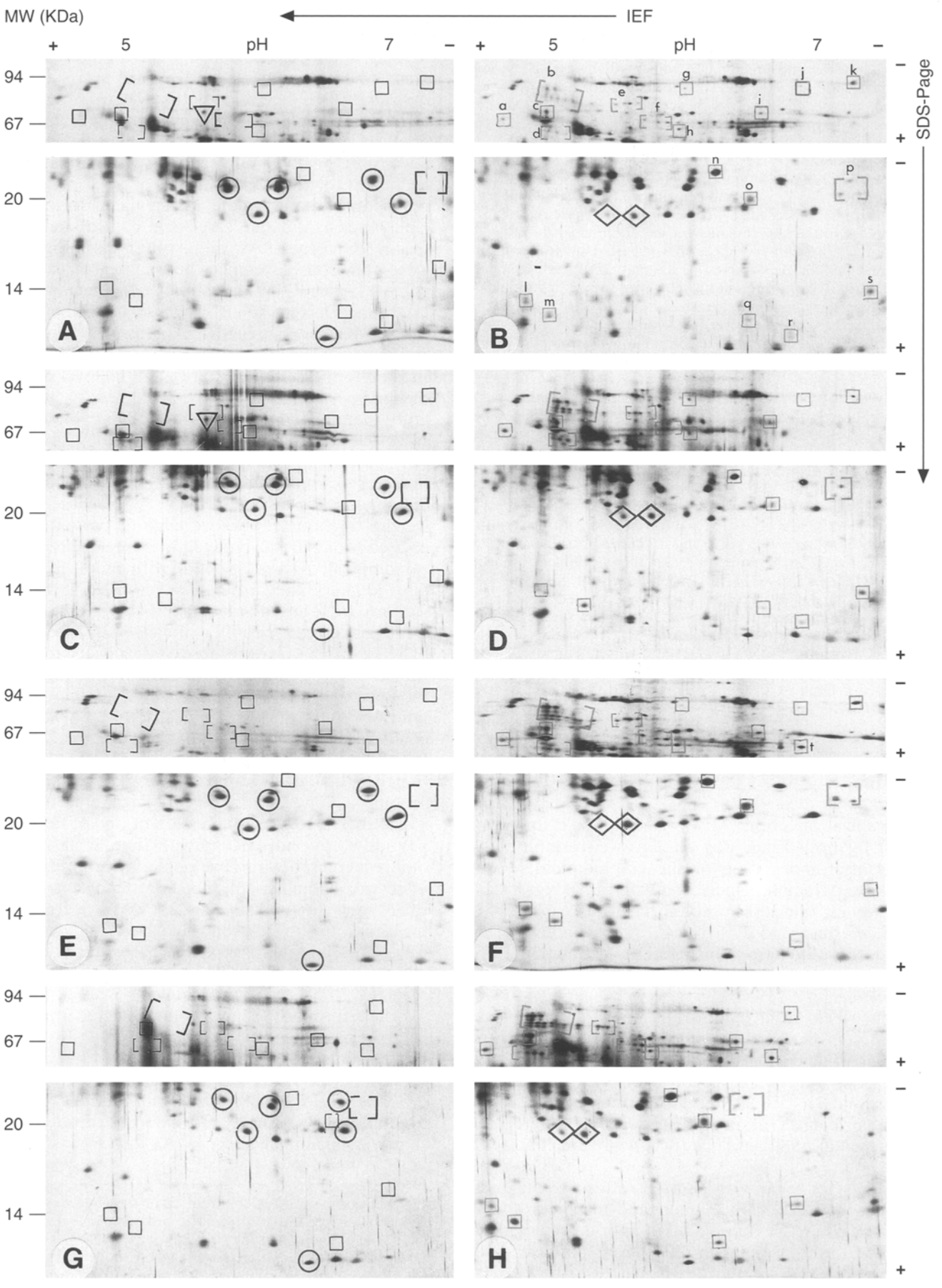
Root sample preparation and mycorrhizal measurements

Control and mycorrhiza-infected tobacco roots were harvested 4, 5, 6 and 7 weeks after sowing, carefully washed in running cold water, weighed, immediately frozen in liquid nitrogen, and stored at −65° C until extraction. At each harvest, samples of the root system were cleared in 10% KOH (30 min, 90° C), stained in 0.1% trypan blue (15 min, 90° C) and the amount of infection estimated according to Trouvelot et al. (1986). The results were expressed as frequency of infected root samples (F%), percent colonized root cortex (M%) and intensity of arbuscule development (A%) within the mycorrhizal root system.

Root extracts and 2D-electrophoresis

Proteins were prepared for isoelectric focussing (IEF) by a phenol extraction method adapted from Hurkman et al. (1989). Briefly, 1 g of frozen roots was ground in liquid nitrogen and the resulting powder was extracted in 10 ml of buffer [0.7 M sucrose,

Fig. 3. Two selected regions from the gels presented in Fig. 2 showing changes with time in protein patterns from uninoculated root extracts of the *N. glutinosa* × *N. debneyi* hybrid (**A, C, E, G**) and of *G. mosseae*-infected root extracts (**B, D, F, H**) after 4 (**A, B**), 5 (**C, D**), 6 (**E, F**) and 7 (**G, H**) weeks of growth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0.5 M Tris-HCl pH 7.5, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2 mM phenylmethylsulfonyl fluoride, 2% (v/v) 2-mercaptoethanol (2-MCE)]. Proteins were separated twice into saturated phenol emulsion and precipitated with 5 volumes of 0.1 M ammonium acetate in methanol. The precipitated proteins were centrifuged, the pellets rinsed with acetone at −20° C, and then dried under nitrogen gas. They were solubilized in 150 µl of the following solution: 9.5 M urea, 4% (v/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 100 mM dithiothreitol, 5% (v/v) ampholytes (1/2 Servalytes (Serva) 3–10, 1/2 ampholytes LKB 5–8), and centrifuged (15 min, 170000 × *g*). Supernatants were stored at −65° C for further analysis. The protein content of the supernatants was determined by the assay of Bradford (1976) with modifications suggested by Ramagli and Rodriguez (1985). The samples loaded onto 2D-PAGE routinely contained 100 µg of protein for mycorrhizal root extracts and 60–90 µg of protein for nonmycorrhizal root extracts, which corresponded to the same fresh weight of roots.

2D-PAGE was carried out according to O'Farrell (1975) with modifications. The first-dimension IEF gels (15 × 0.1 cm) consisted of 9.5 M urea, 0.5% (v/v) Nonidet P 40, 27 mM CHAPS, 4.73% (v/v) acrylamide, 0.27% (v/v) N-N′ bis-acrylamide, 5.5% (v/v) ampholytes (4/5 LKB 5–8; 1/10 LKB 3.5–10; 1/10 Servalytes (Serva) 3–10), 0.1% temed and 0.2% ammonium persulphate. The lower reservoir was filled with 10 mM H_3PO_4, and the upper reservoir with 100 mM NaOH. In each IEF electrophoresis, the range of pH was determined according to O'Farrell (1975). After IEF (15 h, 1000 V), the gels were extruded and equilibrated by incubation in 70 mM Tris-HCl pH 6.8, 5% sodium dodecylsulphate (SDS), 5% (v/v) 2-MCE and 0.3% bromophenol blue for 20 min and then in the same buffer containing 270 mM iodoacetamide (Görg et al. 1987). The second-dimension SDS gels contained 12% acrylamide. Proteins in gels were stained according to Blum (1987). Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa) were used as molecular weight standards (LMW calibration kit, Pharmacia). Each experiment was repeated twice with at least two 2D-PAGE gels per sample.

Results

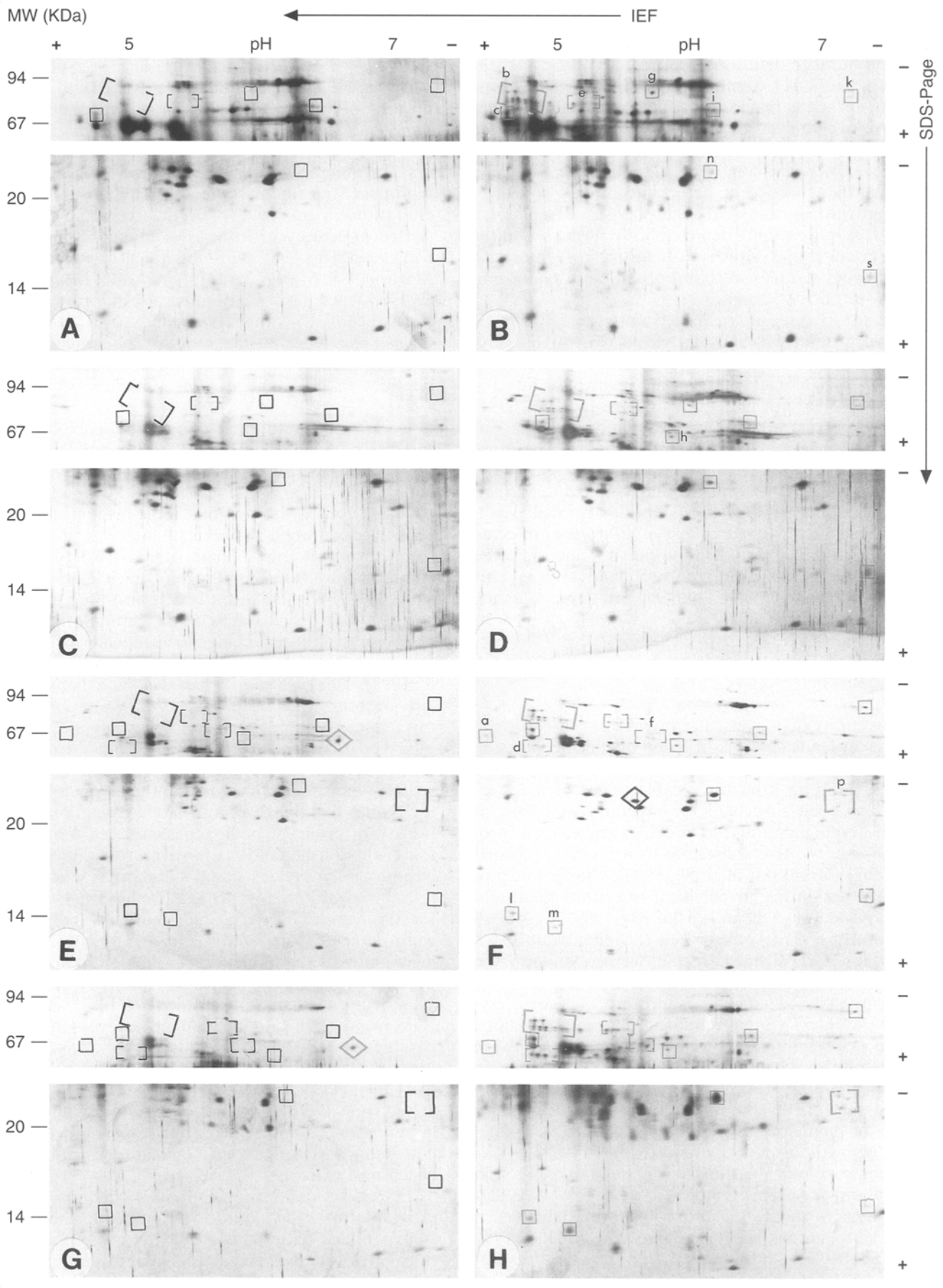
Mycorrhizal infection (M%) and arbuscule formation (A%) by both fungi was already well established 4 weeks after inoculation of the amphidiploid hybrid, and these parameters increased with time (Fig. 1A,B). At 7 weeks, the percent colonized root reached 85% for *G. mosseae* and 79% for *G. intraradices* while arbuscule development, which reflects a functional mycorrhiza, was 65% with *G. mosseae* and 70% with *G. intraradices*.

Four weeks after inoculation, no difference in protein content between the amphidiploid hybrid control and mycorrhizal root extracts was observed, while after 7 weeks of infection mycorrhizal root extracts had an approximately 25% higher protein content than nonmycorrhizal ones, as previously reported for tobacco (Dumas et al. 1989) and pea (Schellenbaum et al. 1992) roots.

2D-PAGE separation showed differences in polypeptides between uninoculated and mycorrhizal root extracts. When the amphidiploid hybrid grew with *G. mosseae,* differences in the 2D-PAGE patterns could be observed at 4 weeks after inoculation (Fig. 2A,B). However, since more than 500 root polypeptides, with molecular sizes ranging from 14 to 94 kDa and pI ranging from pH 4.5 to 7.5, could be resolved by this analytical method, we focussed our attention on two main areas where pronounced differences were evident in the 2D-PAGE. One area, in the upper part of the gels, included polypeptides with apparent molecular weights of 65–95 kDa and pIs of 4.5–6.5. The other area, in the lower part, involved polypeptides with smaller molecular weights of 12–25 kDa and pIs of 5–7.5. We followed polypeptide modifications occurring in these two regions after 4, 5, 6 and 7 weeks in control and *G. mosseae*-infected roots. As can be observed in Fig. 3, there were numerous differences between extracts of uninoculated (A, C, E, G) and *G. mosseae*-infected (B, D, F, H) roots. Despite some variation in the intensity of staining between successive experiments, the effects of endomycorrhizal infection on polypeptide patterns showed four different features: (*1*) the level of most of the polypeptides appeared unchanged, as judged by both the dimensions and the staining intensities of spots (circled spots, for example, in Fig. 3); (*2*) some polypeptides appeared enhanced by mycorrhiza formation (for example, the two polypeptides indicated by a diamond mark in Fig. 3B, D, F, H, with molecular weights of about 20 kDa, were faintly present in uninoculated root extracts but clearly increased in mycorrhizal samples); (*3*) a polypeptide with a molecular weight of 67 kDa normally present in control roots, decreased with mycorrhization (triangle in Fig. 3A,C); (*4*) finally, some novel polypeptides indicated by squares or brackets in Fig. 3 were observed only in mycorrhizal root extracts. After the 4th week of contact between tobacco roots and *G. mosseae,* at least 11 new polypeptides or groups of polypeptides (lettered a–k in Fig. 3) could be detected in the upper part of the 2D-PAGE (molecular weights 65–95 kDa), while 10 polypeptides (labelled l–s) were found in the parts of gels representing a lower range of molecular weights (12–25 kDa). The level of expression of one of these (polypeptide n) did not change with time, while all of the others increased with development of the mycorrhizal infection. The polypeptide r, faintly present in mycorrhizal roots at 4 and 5 weeks, showed a decrease in expression after 6 weeks but increased again at 7 weeks. The polypeptide q disappeared early and could not be observed after 5 weeks when the intensity of infection reached 70%. A polypeptide (t) with a molecular weight of about 65 kDa was only detected after 6 and 7 weeks.

The same time-course analysis of polypeptide profiles was made for root extracts from the amphidiploid tobacco hybrid colonized by *G. intraradices,* compared to uninoculated roots, and we concentrated on the same regions in gels (Fig. 4). As for *G. mosseae* infec-

Fig. 4. Two selected regions from silver-stained two-dimensional gels showing changes with time in protein patterns of uninoculated root extracts from the *N. glutinosa* × *N. debneyi* hybrid (**A, C, E, G**) and of *G. intraradices*-infected root extracts (**B, D, F, H**). For explanation of lowercase letters, see discussion of Fig. 4 in "Results"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tion, root colonization by *G. intraradices* induced novel polypeptides (Fig. 4B, D, F, H). However, they were fewer in number than those observed with *G. mosseae* (Fig. 3B, D, F, H). Some of them displayed the same electrophoretic behaviour as those found in extracts from roots colonized by *G. mosseae* and are indicated by the same letters in Fig. 4 (a–i, k–n, p, s). However, their level of expression varied greatly with time, and some of them were not detectable after 4 weeks of plant growth. Furthermore, the polypeptides denoted a, d, f, l, m, p were only detected after 6 weeks, and the polypeptides j, o, q, r previously found in *G. mosseae*-infected root samples were not observed in *G. intraradices*-infected root samples.

The polypeptide n was only faintly detected in *G. intraradices*-infected roots at 4 weeks (Fig. 4B) and increased subsequently, while it was already present at 4 weeks in a significant amount in *G. mosseae*-infected samples and did not increase with time (Fig. 3B, D, F, H). The polypeptide k (approximately 90 kDa) was still present after 7 weeks of infection by *G. intraradices* (Fig. 4H) whereas it was no longer detectable at the same time in *G. mosseae*-infected root extracts (Fig. 3H). The group of polypeptides termed p was only present after 6 weeks in *G. intraradices*-infected roots (Figs. 4F, H). Finally, one polypeptide indicated by diamond marks on Figures 4F and H was only found in electrophoretic patterns of late infection stages by *G. intraradices.*

Discussion

The possibility that arbuscular mycorrhiza formation in root tissues leads to the expression of specific genes, in response to the morphological and physiological changes resulting from the establishment of the symbiosis, has been suspected for several years. Since it was observed that tobacco roots produced new proteins in response to colonization by arbuscular mycorrhizal fungi (Dumas et al. 1989, 1990), changes in protein patterns during mycorrhizal infections have also been reported in soybean (Packovsky 1989; Wyss et al. 1990) and more recently in pea (Schellenbaum et al. 1992). However, all these results were obtained from 1D-PAGE analysis, with the well-known possibility that many proteins comigrate and thus that some mycorrhiza-induced protein modifications may have been masked, especially where total protein extracts were analysed. Furthermore, the study of changes in protein patterns resulting from arbuscular mycorrhizal infections is still difficult due to the low level of expression of most of them, and the inability to culture the fungal symbionts without the host tissue.

To improve our investigations of gene expression in arbuscular mycorrhizal symbioses, we developed a 2D-PAGE system to analyse the polypeptide patterns of *Xanthi* nc tobacco roots after *G. mosseae* infection (Tahiri-Alaoui 1992). In the present report, using the amphidiploid hybrid *N. glutinosa* × *N. debneyi* (Ahl and Gianinazzi 1982; Ahl et al. 1983), we provide new evidence for modifications in protein patterns linked to the establishment of the mycorrhizal symbiosis. By studying the evolution with time of 2D-PAGE protein patterns, we have been able to demonstrate that colonization of the root system by *G. mosseae* and *G. intraradices* affects the synthesis of polypeptides in the following ways: some polypeptides are not affected, others are enhanced upon mycorrhiza formation, some disappear, and finally some are newly induced. Similar alterations in 2D-electrophoretic profiles have been reported in ectomycorrhizal associations between *Eucalyptus globulus* and *Pisolithus tinctorius* (Hilbert and Martin 1988; Martin and Hilbert 1991). Moreover, recent evidence has been obtained by in vitro translation of total root RNA that colonization of *Allium cepa* by *G. mosseae* leads to the specific appearance of eight new polypeptides and the disappearance of seven polypeptides in mycorrhizal roots (Garcia-Garrido et al. 1993).

So far, we do not have enough evidence to identify the origin of the symbiosis-specific polypeptides in arbuscular mycorrhiza ("endomycorrhizins") since they could be of fungal origin or part of a host response. We have previously reported that some proteins in tobacco roots induced upon mycorrhizal infection are different from the well-known pathogenesis-related proteins stimulated by fungal pathogen attack (Dumas et al. 1989, 1990). We also recently demonstrated that tobacco roots may produce new acidic chitinase isoforms in response to arbuscular mycorrhizal fungi which differ from those induced upon pathogen attack (Dumas et al. 1992). This provides further evidence that some specific proteins must be produced during the arbuscular mycorrhizal symbiosis. Some of the new polypeptides observed after 2D-PAGE could be related to a general stress reaction of the amphidiploid hybrid roots, and we have indications that some of the polypeptides from the groups b and d share a serological relationship with the PR-b_1 group of pathogenesis-related tobacco proteins (unpublished data). However, some of the newly-induced polypeptides could be more specifically related to symbiotic mycorrhiza establishment. Support for this comes from the fact that new polypeptides were detected with both *G. mosseae* and *G. intraradices* and that the induction of some of them was later during infection by *G. intraradices* than by *G. mosseae.* This delayed appearance seemed to be linked to the state of mycorrhizal development as estimated by the parameters M% and A%, which were always lower in *G. intraradices*-colonized roots. On the other hand, a few polypeptides observed early in *G. mosseae*-infected roots were only transiently present, whilst polypeptides with a similar electrophoretic behaviour were detected at a later time in *G. intraradices*-infected tobacco root extracts.

In conclusion, using 2D-PAGE analysis we have provided further evidence for modifications in protein (polypeptide) patterns induced by arbuscular mycorrhizal infection of roots. This is in agreement with previous reports of modifications in protein metabolism following arbuscular mycorrhizal infection of tobacco,

onion, soybean and pea (Dumas et al. 1989, 1990; Packovsky 1990; Wyss et al. 1990; Schellenbaum et al. 1992; Garcia-Garrido et al. 1993). In order to more clearly determine which polypeptides are specific to the arbuscular mycorrhiza symbiosis, 2D-PAGE analyses are presently being compared after root infection by different mycorrhizal and pathogenic root fungi.

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