Research Note

Cytoskeletal Components in the Arbuscular Mycorrhizal Fungus *Glomus mosseae*

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In *Glomus mosseae* microtubules and microfilaments were visualized by indirect immunofluorescence microscopy in hyphae quick-frozen, freeze-substituted, and treated with cell-wall-degrading enzymes. Microtubules were distinguished in both the cortical and the central parts of hyphae, while microfilaments were revealed only in the cortical part. In immunoblotting, tubulin and actin were detected in an extract from hyphae elicited by host plants.

Additional keywords: hyphal growth and morphogenesis.

The spores of arbuscular mycorrhizal fungi can germinate and grow for a limited period in pure culture, but association with plant roots is vital for continuous growth (Siqueira *et al.* 1985; Mosse 1988; Gianinazzi-Pearson *et al.* 1989; Giovannetti *et al.* 1993a). Recently, by utilizing a barrier between the fungus and the host root, it was shown that growth and differentiation of hyphae are elicited by the root without direct attachment between the two partners (Giovannetti *et al.* 1993b). In the present work we used both spore cultures and elicited hyphal cultures to investigate the presence of cytoskeletal elements in hyphae of the arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe.

In fungi, nuclear division and nuclear migration are processes mediated by microtubules (MTs) (Morris and Enos 1992), while apical growth of hyphae is considered to be a process mainly dependent on microfilaments (MFs) (Heath 1990). Cytoskeletal elements are also known to be involved in fungal morphogenesis (cf. Kwon *et al.* 1991a,b). In arbuscular mycorrhizal fungi the factors regulating hyphal growth and appressorium development are still poorly known (Giovannetti and Citernesi 1993). The methods developed in the present work for the visualization of cytoskeletal components may be useful in the future for revealing factors involved in hyphal growth and the differential morphogenesis leading to the formation of infection structures in arbuscular endophytes.

Germinated spores and sporocarps from *G. mosseae* were used. The spores were germinated for 4–6 weeks on dialysis membranes overlying a 1% water agar medium (Hepper and Smith 1976; Giovannetti *et al.* 1991), and the sporocarps were germinated in the presence of roots of the host plants *Ocimum basilicum* L. and *Trifolium repens* L. according to the Millipore sandwich method described by Giovannetti *et al.* (1993a).

The germinated spores and sporocarps were prepared for indirect immunofluorescence (IIF) microscopy by the quick-freezing and low-temperature fixation method described by Raudaskoski *et al.* (1991), except that the samples were fixed with 3.7% formaldehyde in methanol. After fixation and rehydration the samples were treated with cell-wall-degrading 0.2% lysing enzymes from *Trichoderma harzianum* (Sigma) and 0.1% chitinase (Sigma) in phosphate-buffered saline (PBS), pH 5.5, usually for 30 min. The enzyme solution contained a protease inhibitor, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). For visualization of MFs the same enzyme solution was used, but the treatment time was extended to 40 min, and more protease inhibitors were added: 2 mM PMSF, leupeptin (50 µg/ml), and pepstatin (20 µg/ml) (Boehringer Mannheim Biochemicals).

After cell wall degradation the samples were rinsed with PBS, pH 5.5 and 7.3, and then labeled with either monoclonal anti-α-tubulin (DM1A, N.356, Amersham), anti-β-tubulin (DM1B, N.357, Amersham), or anti-actin antibody (N.350, Amersham) diluted 1:500 in PBS buffer, pH 7.3, for 60 min at room temperature or overnight at 4° C. These antibodies have been shown to interact with plant and fungal tubulins and actin (Runeberg *et al.* 1986; Raudaskoski *et al.* 1987; 1991; Lancelle and Hepler 1989; Salo *et al.* 1989; Tang *et al.* 1989; Timonen *et al.* 1993). After the first antibody the samples were rinsed carefully and then treated with the secondary rhodamine-conjugated anti-mouse immunoglobulin G (IgG) antibody (Cappel) diluted 1:40 in PBS buffer, pH 7.3, for 60 min. The samples were rinsed with PBS buffer, pH 8.5, and mounted in glycerol/PBS (1:2), pH 8.5, containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) (1 µg/ml) for visualization of nuclei (Cooke *et al.* 1987). The samples were examined in a Leitz Dialux 20 microscope equipped with appropriate epifluorescence filters.

The quick-freezing and freeze-substitution method was necessary in order to visualize the cytoskeletal elements by IIF microscopy in *Glomus* hyphae, since no success was obtained with conventional fixation in spite of several trials. In contrast to the findings of a previous study (Raudaskoski *et al.* 1991) an enzymatic digestion of the cell wall had to be

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done after the freeze substitution, to enable the antibodies to penetrate the thick, multilayered cell wall typical of arbuscular mycorrhizal fungi (Sward 1981a,b; Bonfante-Fasolo and Schubert 1987; Giovannetti *et al.* 1991; Meier and Charvat 1992). The preservation of intact MTs seemed to be dependent on the extent of the enzyme treatment. Fragmented MTs always occurred when the hyphae were submitted to too long or too strong an enzyme treatment. When hyphae consisting of a thin and a thick part were treated with the enzyme solution for an extended period, the MTs of the thin hyphae were

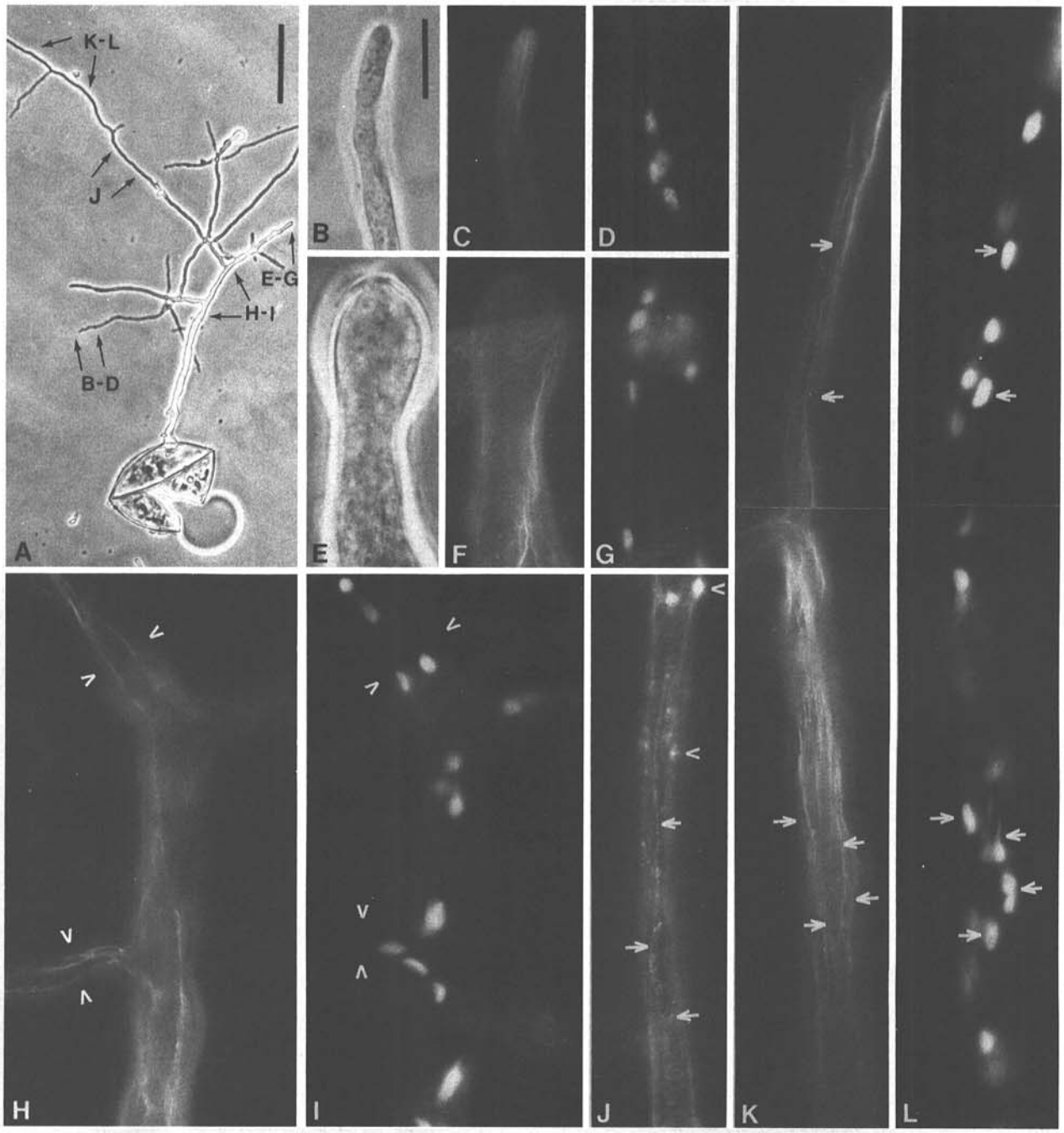

Fig. 1. Phase-contrast and fluorescence microscopy of the microtubule (MT) cytoskeleton (**C, F, H,** and **K**), the microfilament (MF) cytoskeleton (**J**), and nuclei (visualized by staining with 4′,6-diamidino-2-phenylindole [DAPI]) (**D, G, I,** and **L**) of *Glomus mosseae* hyphae originating from spores germinated *in vitro*. The MT cytoskeleton is visualized with antibodies against α-tubulin (**C, F,** and **K**) and β-tubulin (**H**). **A,** General view of *G. mosseae* hyphae after freeze substitution. The letters mark hyphal parts corresponding to those presented in **B–L. B,** Phase-contrast micrograph of a hyphal tip; **C,** MTs extending close to the apex of the hypha; **D,** nuclei visualized by DAPI staining (nuclei are absent from the tip of the hypha). **E,** Phase-contrast micrograph of the apex of a thick subtending hypha; **F,** MTs beneath the plasma membrane of the hypha; **G,** nuclei visualized by DAPI staining. Note the differences in the locations of the nuclei in the thick hypha (**G**) and the thin hypha (**D**). **H,** Continuation of MTs from a thick main hypha into the branches formed by thin hyphae (arrowheads); **I,** nuclei of this hyphal system visualized by DAPI staining. **J,** MFs, actin dots (arrows), and plaques (arrowheads) in the cortical part of an elicited hypha. **K,** Intact MTs in a thin hypha; **L,** the corresponding nuclei visualized by DAPI staining; the close association of MTs and nuclei in the hypha are marked by arrows. Bar in **A** = 100 μm; bar in **B** = 10 μm.

fragmented, but those of the thick hyphae remained intact, probably since the thick wall protected the MTs from proteolytic enzymes (data not shown). For MF visualization the cell wall degradation time had to be increased in order to enable the penetration of the IgM-class anti-actin antibody into the hyphae. In this case the MFs were protected by an increase in the concentration of protease inhibitors in the wall-degrading enzyme solution.

The MTs were visualized equally well with the α- and the β-tubulin antibodies (Fig. 1C, F, H, and K), and they were detected both in the subtending thick hyphae and in the thin hyphae emerging from them (Fig. 1C, F, H, and K). MTs were located in both the cortical and the central parts of the hypha (Fig. 1F, H, and K). In the hyphal apex, MTs extended to the extreme tip (Fig. 1C), which suggested that in *G. mosseae* hyphae not only MFs (Heath 1990) but also MTs might be involved in hyphal tip growth, as has been suggested in some filamentous fungi (Howard and Aist 1977, 1980; M. Raudaskoski, unpublished results). No nuclei could be seen close to the tips of thin hyphae (Fig. 1D), in agreement with findings reported earlier for *Gigaspora* (Cooke *et al.* 1987). MTs continued from the main hyphae into the branches (Fig. 1H and K), and the positions of nuclei (Fig. 1I and L) appeared to follow the MT tracks. The close association of MTs and nuclei indicated that the distribution of nuclei in the hypha could be an MT-dependent process. In an electron microscope study of *Gigaspora* (Sward 1981b) MTs were also seen in close association with nuclei and mitochondria. MT cytoskeletons in hyphae grown on water agar appeared to be similar in structure to those in hyphae elicited by host plant roots. No spindles of dividing nuclei were detected in hyphae of *Glomus*, although nuclei have recently been reported to divide in outgrowing *Gigaspora* hyphae *in vitro* (Bécard and Pfeffer 1993).

IIF microscopy of actin in *Glomus* succeeded only in the elicited hyphae (Fig. 1J). In *G. mosseae* actin was mainly resolved in MF-like structures, as in the oomycete *Saprolegnia ferax* (Heath 1987). In contrast to the MTs, the MFs seemed to be located only in the outermost layer of the cytoplasm beneath the plasma membrane (Fig. 1J). In addition to the MFs, actin plaques and small dots were also seen in G. *mosseae*, as in *S. ferax* (Heath 1987) and in a species of the zygomycete *Neozygites* (Butt and Heath 1988). It has been suggested that the plaques are filasomes, vesicles associated with actin (Hoch and Staples 1983, 1985).

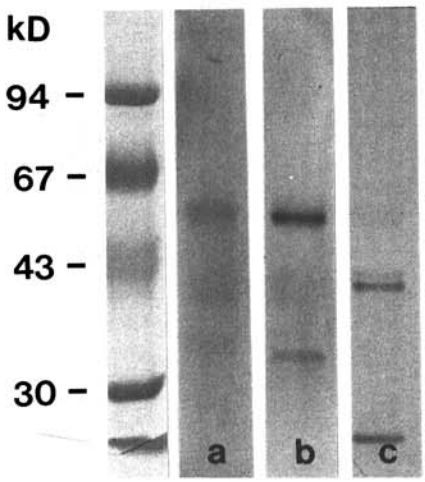

Fig. 2. Immunoblots of α-tubulin (lane a), β-tubulin (lane b), and actin (lane c) from elicited hyphae of *Glomus mosseae*. The same extract and sample volume were used in each lane. According to the protein determination, the total protein content in each lane should be 19 μg, which is probably too high, because of impurities in the extract that disturbed the measurement. Low molecular weight breakdown products occur in lanes b and c. On the left are molecular weight markers stained with amido black.

For immunolabeling experiments the small amount of germinated material available was collected as fast as possible in 20 μl of extraction buffer. The buffer contained 100 mM Tris-HCl (pH 8.5), 20% glycerol, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate (Hurkman and Tanaka 1986), and the following protease inhibitors: leupeptin (25 μg/ml), pepstatin (25 μg/ml), aprotinin (10 μg/ml), α_2-macroglobulin (1 U per 3 ml) (Boehringer Mannheim Biochemicals), 1 mM benzamidine HCl, and 1 mM PMSF (Sigma) (Drubin *et al.* 1988). The spores, sporocarps, and hyphae were carefully crushed with forceps, 30 μl of extraction buffer was added, and then the sample was frozen in liquid nitrogen. The crushing and freezing was repeated several times, and the breakdown of the fungal material was followed with the microscope. After the extraction protein determination, electrophoresis and immunoblotting were done as described earlier (Raudaskoski *et al.* 1987; Åström *et al.* 1991). The same tubulin and actin antibodies were used as in the IIF microscopy (Fig. 2). The immunoblots from germinated spores succeeded only with β-tubulin (results not shown), but the extraction of the elicited cultures gave a protein yield high enough for immunodetection of both tubulins and actin. This was probably due to the larger amount of hyphae available in the elicited cultures than in the spore cultures. In immunoblots with the same amount of extract from the same sample of elicited hyphae per well, the tubulin antibodies revealed a weak α- and a strong β-tubulin band with almost similar mobility and M_r of 52–56 × 10^3 (Fig. 2, lanes a and b). In the immunoblots of fungal proteins the antibody against β-tubulin gave a stronger signal than that for α-tubulin, probably because α-tubulin has a poorer capacity for binding to its antibody than β-tubulin does (cf. Salo *et al.* 1989; Timonen *et al.* 1993). This difference is usually seen when the protein content of the sample is low, and it could explain why a β-tubulin signal but not an α-tubulin signal was obtained in the extracts from germinated spores. The actin antibody revealed two close bands, a weak and a strong one, with M_r of 43 × 10^3, typical of actin (Fig. 2, lane c). Whether these two bands represent two different actins, or whether the lower band is a proteolytic breakdown product of the higher one, has to be resolved in the future. In the actin but not in the tubulin blots, a strong staining of the protein front at the bottom of the gel was also seen. This was interpreted to be the result of a breakdown of actin. The immunodetection of actin from filamentous fungi was significantly improved (cf. Salo *et al.* 1989; Timonen *et al.* 1993) when protease inhibitors used were the same as those used in the extraction of actin from yeast cells (Drubin *et al.* 1988). In the present work these inhibitors were not sufficient, perhaps because of the slow extraction procedure and the high proteolytic activity in the *Glomus* hyphae cultured *in vitro*. This was also indicated by the occurrence of a weak band of lower molecular weight in the β-tubulin immunoblot.

The present results show that the immunological methods developed for investigation of the cytoskeleton in filamentous fungi may also be applied to arbuscular mycorrhizal fungi.

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