Mycorrhiza (1996) 6:423–429 © Springer-Verlag 1996

ORIGINAL PAPER

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Dynamics of cytoskeletal proteins in developing pine ectomycorrhiza

Accepted: 6 May 1996

Abstract Mycorrhizal short roots of *Pinus contorta* Dougl. ex Loud colonized by *Suillus variegatus* (Sow. ex Fr.) O. Kuntze or *Paxillus involutus* (Batsch) Fr. were collected 1–>60 days after fungal contact. The proteins of the inoculated roots were extracted, electrophoretically separated, blotted and immunostained for α-tubulin and actin. The development of the mycorrhiza was also followed microscopically. The signal of plant α-tubulin was stronger than the signal of fungal α-tubulin during the first 5 days in *S. variegatus* mycorrhiza and was then exceeded by fungal α-tubulin. This correlated well with the increase of fungal mycelium in the mycorrhiza. A transient drop in both plant and fungal α-tubulin signals was observed 20 days after fungal contact, suggesting a change in the metabolism of the mycorrhiza. The signals for plant and fungal actins in the mycorrhiza increased steadily during early infection and then remained at a high level as the mycorrhiza matured. Similar trends were observed in *P. contorta–P. involutus* mycorrhiza. The data from *P. contorta–S.variegatus* mycorrhizas suggests that α-tubulin is a growth-related protein, subject to changes, while the amount of actin reflects the general metabolic activity of the mycorrhiza. In both mycorrhizal systems clear α-tubulin and actin signals were detected 60 days after colonization, which indicates that the mycorrhizas were metabolically active in spite of their withered appearance.

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Key words Actin · Mycorrhiza · *Pinus contorta* · *Suillus variegatus* · Tubulin

Introduction

During ectomycorrhiza formation both plant cells and fungal hyphae show developmental patterns which differ from those observed during nonsymbiotic growth (Nylund and Unestam 1982; Kottke and Oberwinkler 1986; Horan et al. 1988; Piché et al. 1988; Peterson and Bonfante 1994, Scheidegger and Brunner 1995). The characterization of the molecular factors and cytoskeletal changes involved in the symbiotic development is in progress (Martin and Tagu 1995). In the symbiotic plant and fungal cells reorganization of the structure of the cytoskeleton has been reported (Timonen et al. 1993). Variation in levels of cytoskeletal proteins of plant and fungal symbionts in the ectomycorrhizal samples was observed. The latter observations could reflect the development of ectomycorrhizas, since the polymerized products of tubulin and actin proteins, microtubules and actin filaments, are known to be essential for growth and differentiation of plant and fungal cells (Heath 1990; Staiger and Lloyd 1991).

In order to obtain a more detailed picture about the functions of both plant and fungal cells during ectomycorrhizal development and about the role of the cytoskeletal elements in this development, the levels of α-tubulin and actin were followed in *Pinus contorta* Dougl. ex Loud.–*Suillus variegatus* (Sow. ex Fr.) O. Kuntze and in *Pinus contorta–Paxillus involutus* (Batsch) Fr. ectomycorrhiza of different ages. α-Tubulin is an especially good tool for this kind of investigation since plant and fungal α-tubulins are electrophoretically separable (Timonen et al. 1993). The changes in the plant and fungal α-tubulin levels may thus give an insight into the activity and dominance of each symbiont at different stages of ectomycorrhizal development. Actin levels were followed to ascertain the metabolic activity of the mycorrhizal roots.

Materials and methods

Mycorrhiza synthesis and harvesting

Ectomycorrhizal associations between *Pinus contorta* and *Suillus variegatus* or *Paxillus involutus* were synthesized aseptically following the method described by Duddridge (1986) and modified by Finlay et al. (1988). After about 6 weeks the mycorrhizal seedlings were transplanted to 15×15 cm^2 Perspex observation chambers (Finlay and Read 1986) on a 2-mm-thick layer of unsterilized *Sphagnum* peat. When the external hyphae had colonized the whole peat surface, uninfected pine seedlings with lateral and short roots were planted on the hyphal mat. Colonization of short roots took place in 1–5 days. The position of each short root was marked with a color code when hyphal contact with the root surface was observed. A stereo microscope was used to follow the overall appearance of the short roots of different infection stages and typical representatives of each age group were photographed. The mycorrhizal short roots were picked individually 1, 3, 5, 7, 10, 20, 30, 40, 50 and >60 days after hyphal contact in *Suillus variegatus* systems (Fig. 1). As reference material, a young, mature and old mycorrhiza time series (5, 20 and >60 days respectively with an extra point at 10 days) was prepared from *Paxillus involutus* mycorrhizas.

Extraction and immunodetection of cytoskeletal proteins

Proteins were extracted from the mycorrhizal roots of certain age by two different procedures. In the first method 50–100 mycorrhizal roots were ground into fine powder in a mortar with liquid nitrogen. The frozen powder was transferred into an Eppendorf tube with 500–700 μl SDS extraction buffer with proteinase inhibitors (Timonen et al. 1993). In the second procedure 15–40 mycorrhizal roots were directly homogenized and extracted in 100 μl SDS extraction buffer with proteinase inhibitors in a 2-ml glass mortar kept in ice. With the latter method the loss of material was small. This allowed a more precise estimation of the relationship between the number of extracted mycorrhiza and amount of protein in the solution. For protein measurements 10 μl of each extract was precipitated and the amount of protein was determined using the Bio-Rad micro assay procedure. Two or three replicate samples were analyzed on 10% SDS-polyacrylamide mini gels in a Bio-Rad Mini-Protean II dual slab cell system. Each well was loaded with a sample calculated to contain 10 μg total protein. This required 80–100 μg dry mycorrhiza, equivalent to three to seven fresh mycorrhizal short roots, the number depending on their size. The mycorrhizal dry weight per short root was determined by collecting 20–40 short roots 0, 5, 10, 20 and 30 days after contact and drying the roots for 12 h at 90 °C. The amount of total protein in the samples of mycorrhizas of different ages remained fairly constant, being approximately 100 μg mg^{-1} mycorrhizal dry weight.

Immunoblotting of the gels were performed as described by Åström et al. (1991). Monoclonal α-tubulin (N356, Amersham) and actin (N350, Amersham) antibodies were used for immunodetection of cytoskeletal proteins. Antibodies were used at concentrations of 1:2500 for α-tubulin and 1:1000 for actin. Peroxidase-conjugated immunoglobulins against mouse immunoglobulins (P 161, Dakopatts A/S) were used as a secondary antibody.

The intensity of the bands in the immunoblots was scanned and measured using a ImageMaster DTS scanner and ImageMaster 1-D software (Pharmacia). The relative differences in staining intensities of the bands were maintained on different nitro-cellulose filters, although overall band intensities obtained were affected by the staining procedure. An internal standard of one extract of 10-day-old mycorrhiza was used to adjust the blots with each other. In the figures α-tubulin and actin were analyzed from 10 μg total protein in each sample. In the α-tubulin blots the lower band is formed by plant α-tubulin and the upper band by fungal α-tubulin (Timonen et al. 1993). As controls, 0.5, 1, 2.5, 5, 10, 15, 20 and 25 μg of protein extracted from *Pinus contorta* radicles and pure culture of *S. variegatus* were electrophoretically separated and immunoblotted with monoclonal antibodies against α-tubulin and actin as described before. The intensity of the signal of both proteins was recorded using ImageMaster 1-D software.

Microscopy

Semi-thin cryosections (10 μm) for light microscopy were produced at each stage of colonization to visualize the changes taking place in the short root. Mycorrhiza of each stage of colonization were fixed with 3.7% formaldehyde in phosphate buffer, infiltrated with cryoprotectants and sectioned with a cryomicrotome (Leitz, Cryostat 1720) at –32 °C in Tissue-Tek embedding medium (Histolab Products). Sections were observed and photographed in order to identify the stage of mycorrhiza formation and to appreciate the relative amount of fungal and plant tissue.

Results

The development of *Pinus contorta–Suillus variegatus* mycorrhiza, from the first contact of hyphae with the root until 50 days after contact, is shown in Fig. 1. From day 3 onwards the root meristem was totally covered by fungal mycelium (Fig. 1c). During the development of the ectomycorrhiza the short roots became dichotomous. The first signs of two tips in the short roots were observed 5 days after contact (Fig. 2d). The sections of the short root, however, showed that the root tip meristem could divide as early as 1 day after hyphal contact (Fig. 2a). The examination of the sections indicated that the meristem of the dichotomous roots could divide further. Short roots with more than two meristems were detected in roots 10 days after contact (Fig. 2b), but in these growth conditions they were rare. The elongation of the new tips occurred mainly between 5 and 30 days after contact (Fig. 1f–h).

In the sections of the short roots which had been in contact with the mycelium for 3 days, a loose thin sheath and some intercellular colonization of the cortex by the hyphae was observed (Fig. 2c). The thickness of the sheath and the colonization of the intercellular space between the cortical cells increased with time. In the samples 20 days old and older, no contents could be seen in the cortical cells in the first vigorously colonized areas (Fig. 2d). The longitudinal sections of the short roots also suggested that the number of meristematic cells in the root tip decreased when the colonization increased. Meristematic tissue was, however, detected even in the oldest mycorrhizal roots examined (Fig. 2e).

The protein controls from *Pinus contorta* radicles and *Suillus variegatus* pure culture immunostained for α-tubulin and actin showed that with both materials and in both treatments the signal intensified as the amount of protein increased (Fig. 3). The α-tubulin and actin antibodies reacted sufficiently similarly with plant and with fungal cytoskeletal proteins to justify their use in detecting occurrence of α-tubulin and actin during ectomycorrhizal development.

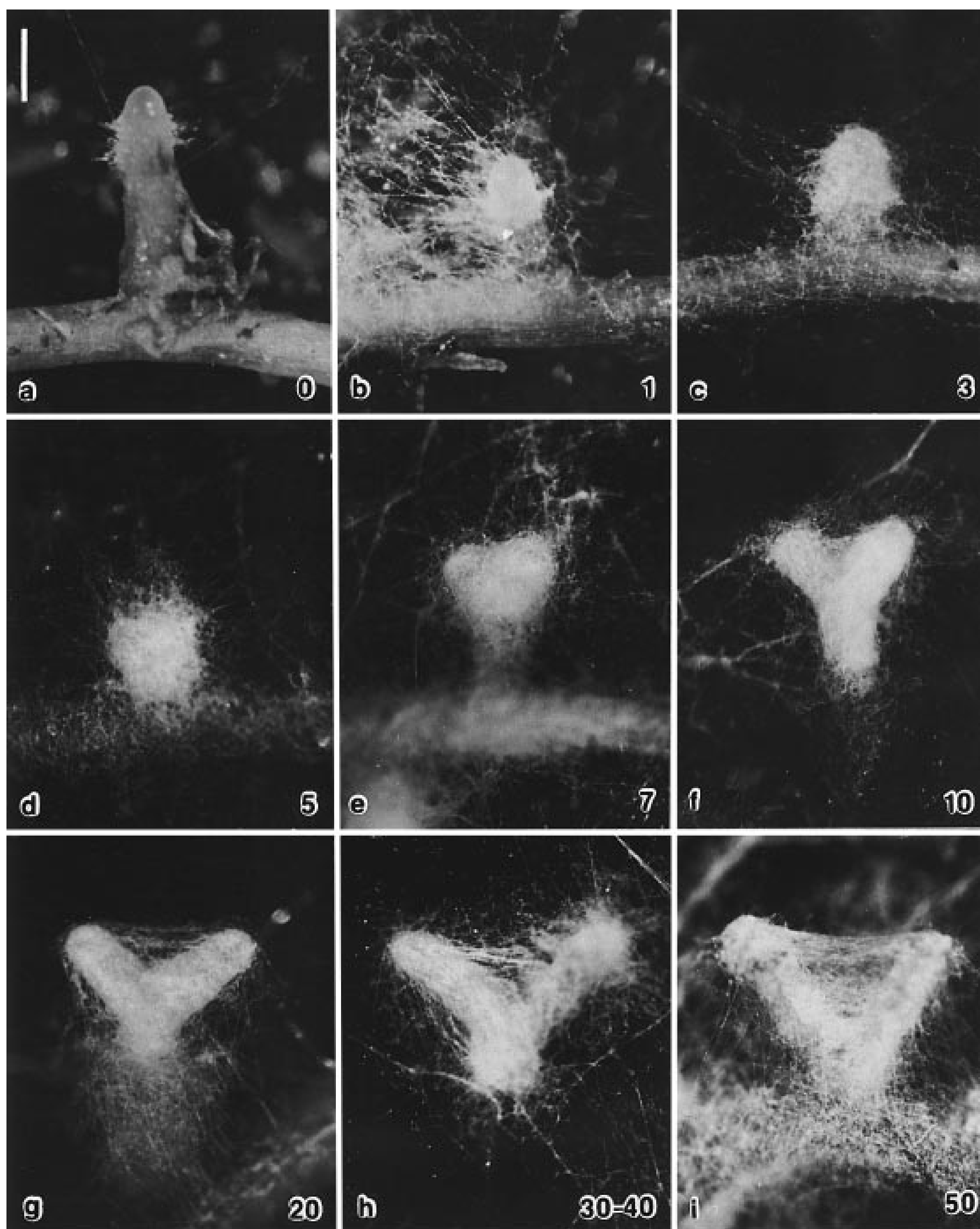

Fig. 1 Typical representativesof *Pinus contorta–Suillus variegatus* mycorrhizas collected for α-tubulin and actin analysis. The number of days (0–>50) since first contact between the short root and the hyphae is given in the right corner of each picture. *Bar* 1 mm

Notwithstanding variability between replicates, the plant α-tubulin signal (lower band) was intense in the mycorrhizal samples collected and extracted 1 and 5 days after fungal contact with the short root, while the fungal α-tubulin signal (upper band) increased from barely detectable in the 1-day sample to a clear signal in the 5-day samples (Figs. 4, 5). In the 7- and 10-day samples the intensity of the fungal α-tubulin signal remained comparable to that in the 5-day sample. In the same extracts the intensity of the plant α-tubulin signal was reduced, probably due to a dilution effect of the increasing amount of fungal proteins in the samples analyzed. A clear reduction of both plant and fungal α-tubulin signal intensity was observed in two separate samples collected 20 days after contact. The reduction was not permanent but was followed by an increase in

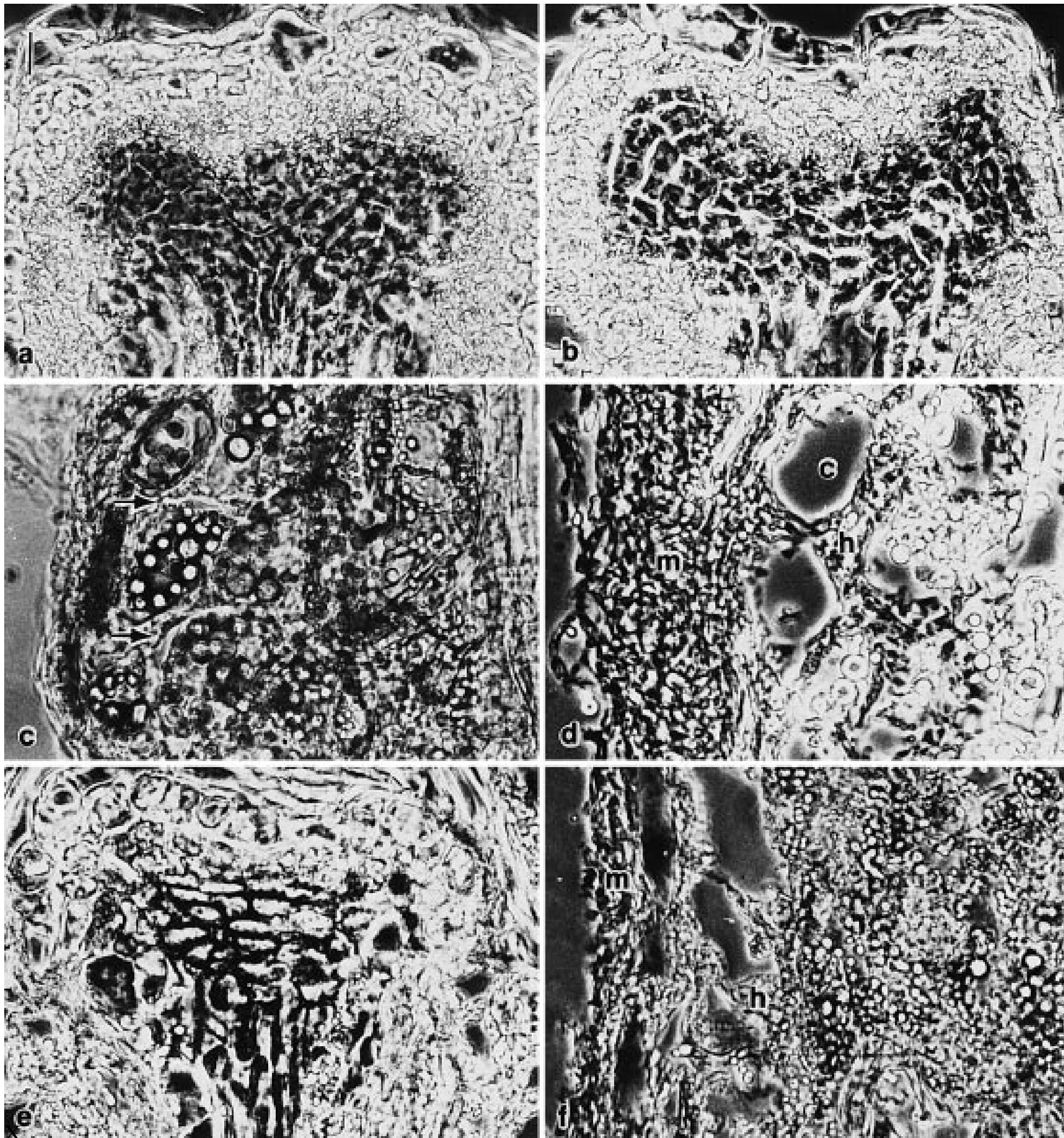

Fig. 2 Cryosections representing differently aged *Pinus contorta–Suillus variegatus* mycorrhizas (**a–e**) and *Pinus contorta–Paxillus involutus* mycorrhizas (**f**). **a** Division of the apical meristem of a short root into a dichotomous mycorrhizal meristem 1 day after fungal contact. **b** Further dichotomization of the meristem in already dichotomous mycorrhizal root, occurring approximately 10 days after hyphal contact. **c** Initial hyphal penetration between cortical cells (*arrows*) 3 days after contact. **d** Thick mantle (*m*) and Hartig net (*h*) 20 days after contact. Note the absence of cytoplasm in certain cells in this area (*c*). **e** Meristematic tissue in mycorrhiza 50 days since hyphal contact. **f** Thin mantle of *Pinus contorta–Paxillus involutus* mycorrhiza (*m*) and slight colonization of cortical tissue (*h*) 20 days after hyphal contact. *Bar* 10 μm

fungal and plant α-tubulins in the 30- and 40-day samples, respectively. In these samples the fungal α-tubulin signal was stronger, while in the 50-day sample the intensity of the plant and fungal α-tubulin signals were comparable and slightly weaker than in the 40-day sample. In the samples older than 60 days the plant and fungal bands were difficult to separate but their intensities were comparable when seen. The amount of total actin in ectomycorrhiza increased during the first 10 days after contact (Figs. 4, 6), and then remained almost the same in all the later samples.

The longitudinal sections of *Paxillus involutus* mycorrhiza of the different ages showed that the sheath and the Hartig net formation was not as rapid as in *Suillus variegatus* mycorrhiza. The colonization of short roots by the hyphae was also never as extensive as by *Suillus variegatus* (Fig. 2f). In *Paxillus involutus* mycorrhiza the signal of plant α-tubulin remained intense to 10 days then declined as the mycorrhiza matured, while the amount of fungal tubulin steadily increased during the first 20 days (Figs. 4, 7a). A 20-day drop in tubulin levels was not observed. In the sample representing mycorrhizal roots >60 days after fungal contact

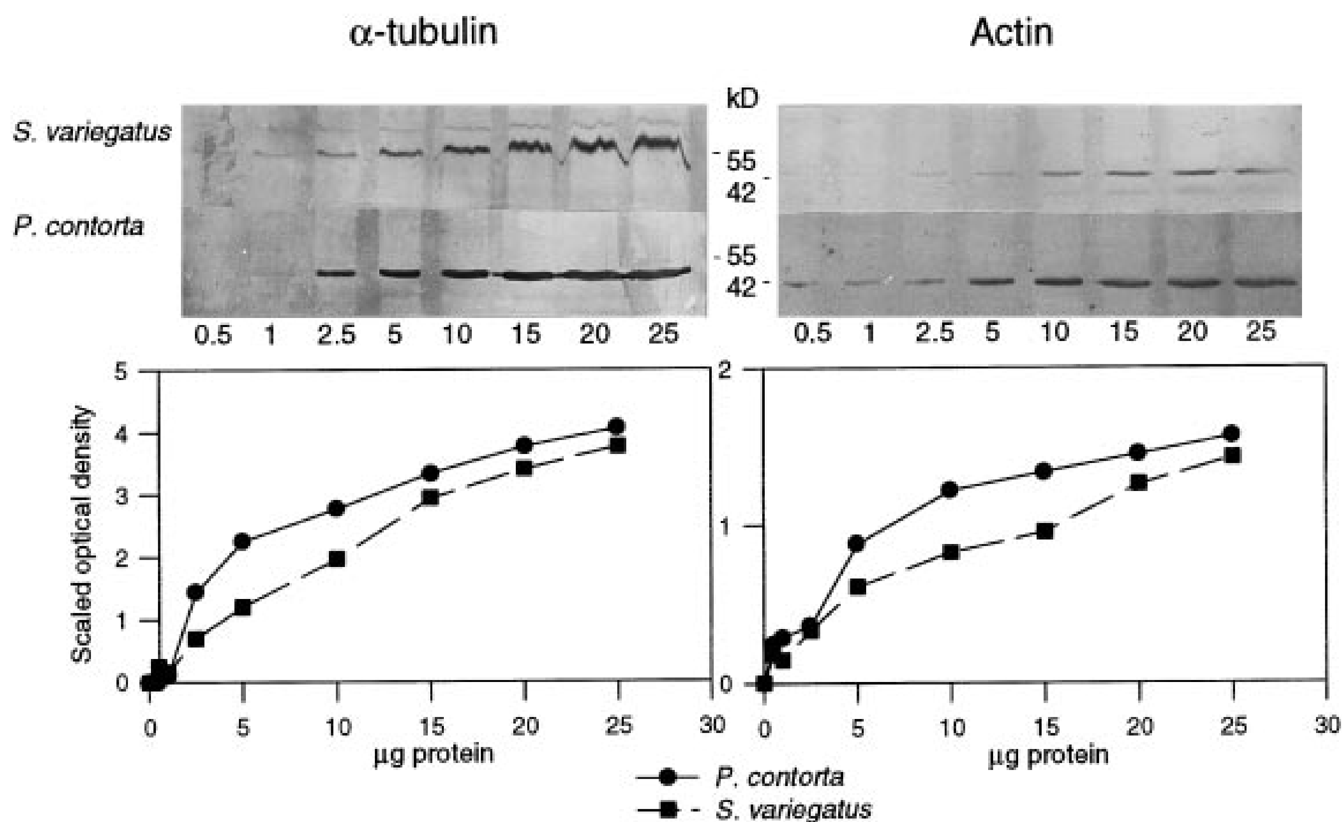

Fig. 3 Immunoblots of 0.5, 1, 2.5, 5, 10, 15, 20 and 25 μg of protein extracted from *Pinus contorta* radicles and a pure culture of *Suillus variegatus* with α-tubulin and actin antibodies, and scaled optical densities of the bands of the immunoblots

the signals given by plant and fungal α-tubulins were similar. The amount of actin remained at approximately the same level in all the studied samples (Fig. 7b).

Discussion

The high levels of plant α-tubulin at the beginning of mycorrhiza formation in both *Pinus contorta* symbioses may be related to the growth and dichotomization of the short roots during the first days of symbiosis. The amount of fungal α-tubulins increased in both *Pinus contorta–Suillus variegatus* and *Pinus contorta–Paxillus involutus* systems over the first 10 and 20 days, respectively. At the same time the initially strong signal of plant α-tubulin declined, probably as a consequence of the higher amount of fungal than plant proteins in the samples. The increase in the amount of fungal tubulin correlated well with the growth of the mycelium in and around the short roots recorded in the microscopical investigations of the roots. In indirect immunofluorescence (IIF) microscopic studies of developing *Pinus sylvestris–Suillus bovinus* mycorrhiza, microtubule tracks and spindles were frequently seen in the hyphae next to the short roots (Timonen et al. 1993). Microtubules are known to be prominent structures also in actively grow-

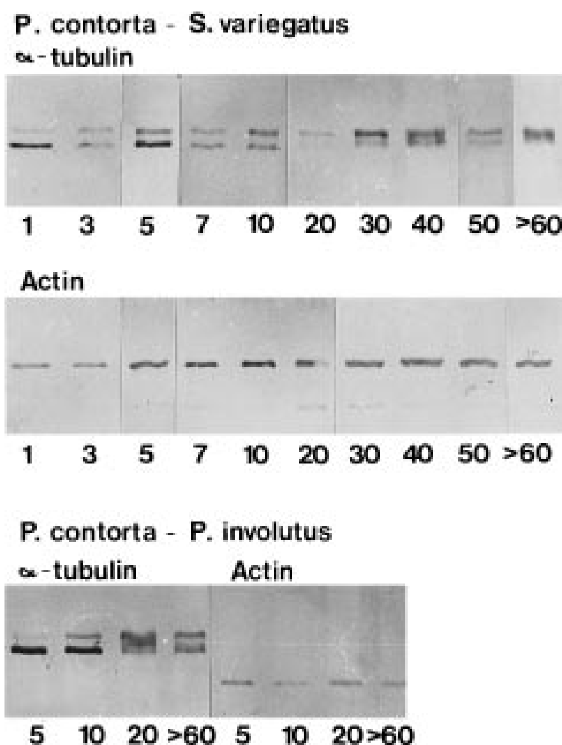

Fig. 4 α-Tubulin and actin immunoblots of *Pinus contorta–Suillus variegatus* and *Pinus contorta–Paxillus involutus* mycorrhizas at different times since contact. The total protein content of each lane is 10 μg. The number of days since contact is given at the bottom of each lane

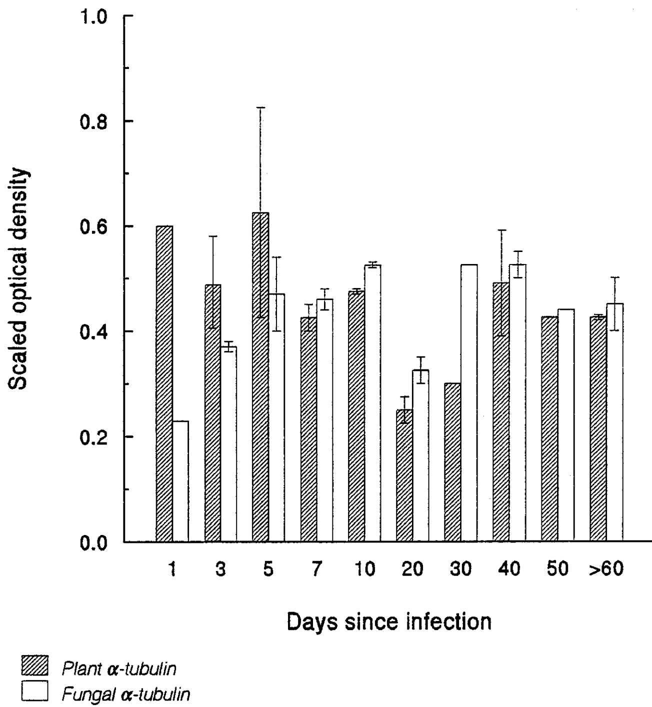

Fig. 5 The intensity of plant and fungal α-tubulin signals per 10 μg protein in *Pinus contorta–Suillus variegatus* mycorrhiza at different times since hyphal contact. The intensities of α-tubulin and actin bands are estimated by measuring the optical densities of the immunoblotted bands. The number of days since hyphal contact is given below each column. The *error bars* give the range of variability for two or three replicate samples

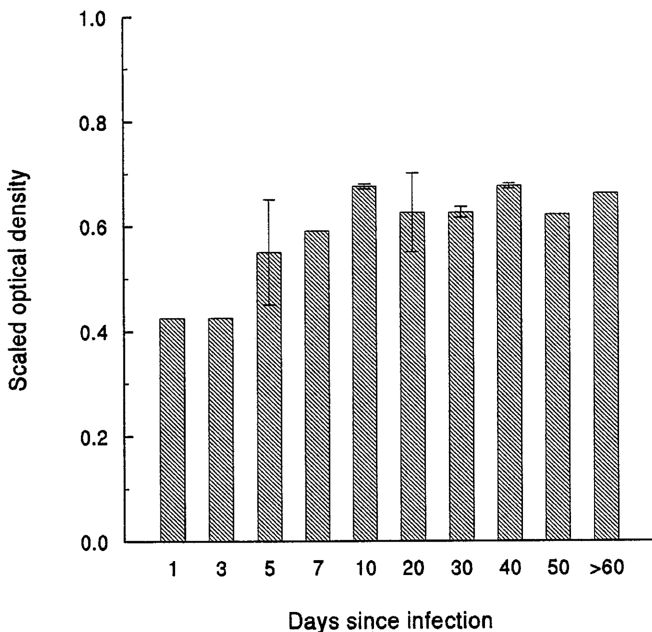

Fig. 6 Intensity of combined plant and fungal actin signals per 10 μg protein in *Pinus contorta–Suillus variegatus* mycorrhiza at different times since hyphal contact, measured as in Fig. 5

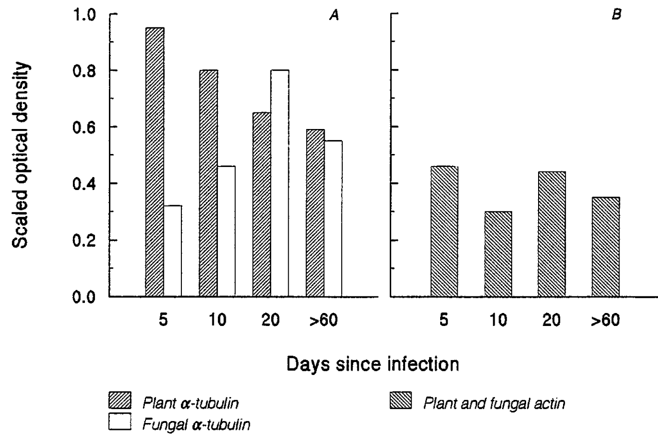

Fig. 7A, B Intensity of plant and fungal α-tubulin and actin signals per 10 μg protein in *Pinus contorta–Paxillus involutus* mycorrhiza at different times since hyphal contact, measured as in Fig. 5

ing apical cells of mycorrhizal fungi in axenic conditions (Salo et al. 1989; Niini and Raudaskoski 1993). This interpretation is in accordance with the observation that in the 5- and ten-day old ectomycorrhiza of *Pinus contorta–Paxillus involutus* the growth of the mycelium was much less extensive than in *Pinus contorta–Suillus variegatus* mycorrhiza, and during that time the plant α-tubulin signal exceeded the fungal one.

In the 20-day samples of *Pinus contorta–Suillus variegatus* mycorrhiza the amount of both plant and fungal α-tubulin was barely detectable, which suggested cessation of growth of both partners. Judging from the increase of the α-tubulin signals in the later samples, the pause in growth was only temporary or the microtubules have functions other than those connected with the growth of fungal hyphae and plant cells. The observation by Timonen et al. (1993) of large amounts of microtubule bundles in the mantle and the shift in microtubule orientation to network-like structures in the Hartig net of pine mycorrhiza would indicate a specialized task for the microtubules in mycorrhizal transport. Microtubules have been shown to interact with membrane proteins of tobacco cells (Akashi and Shibaoka 1991; Laporte et al. 1993; Sonobe and Takahashi 1994), so it is possible that microtubules could affect the translocational routes of the symbiotic cells and the positioning of membrane proteins.

The collecting criterion of the short roots was age, not their exact morphological appearance; therefore, mycorrhiza at slightly different developmental stages were probably included in a single sample. This probably explains the variation in the detection of plant α-tubulin between the 5- and 40-day samples. At those sampling times dichotomization or regrowth may have been initiated in some root tips, while in other tips the start of growth lagged behind or did not occur. Downes et al. (1992) reported a similar type of diversity in the development of spruce mycorrhizas. They observed pe-

riodic bursts of growth in some but not in all mycorrhizas, which resulted in diverse appearance of mycorrhizal short roots of the same age.

The levels of actin appeared stable in the same samples in which the α-tubulin levels varied. Partly this was due to the similar mobility of plant and fungal actin in 1-D electrophoresis. The reduction of actin level in one of the partners could be obscured by the increase of actin in the other. However, no reduction comparable to that of α-tubulin was observed in the actin level in the 20-day samples. This supports the idea that the levels of tubulin detected in the symbiotic partners reflect the events associated with growth more than those of actin. Clear actin signal in addition to the tubulin signals in the mycorrhizal short roots over 60 days old is in agreement with the previous reports, which have shown that in favorable conditions mycorrhizal roots can survive for as long as 2 years (Robertson 1953).

On the basis of the present work it appears that the variation in tubulin levels could be associated with the growth of the partners in the ectomycorrhiza, while the actin signals could reflect the metabolic activity of the ectomycorrhizal root. IIF microscopy in time-course studies has already revealed that the cytoskeleton plays a role during pathogenic fungal invasion of the plant tissues (Kobayashi et al. 1991; Kwon et al. 1991). The changes in plant and fungal cells at specific times during mycorrhizal development still need to be visualized with IIF or immuno-electron microscopy.

Acknowledgements We wish to thank Robin Sen and Helena Åström for critical reading of the manuscript and Jan Lindström for technical assistance in drawing Figs. 4–6. Financial support from the Nordic Joint Committee for Agricultural Research is gratefully acknowledged.

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