Protoplasma (1995) 188: 161–169

PROTOPLASMA

Analysis of the cell cycle in an arbuscular mycorrhizal fungus by flow cytometry and bromodeoxyuridine labelling

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Received December 30, 1994
Accepted February 13, 1995

Summary. The cell cycle of an arbuscular mycorrhizal fungus, *Glomus versiforme*, was determined by flow cytometric analysis of nuclei isolated from spores and mycorrhizal roots of leek, and by immunogold staining after bromodeoxyuridine (BrdU) uptake by DNA. The aims of our work were to establish: (*i*) whether there are changes in ploidy during fungal growth and morphogenesis, (*ii*) when and where the cell cycle is activated. Our results demonstrate that nuclei isolated from quiescent spores of *G. versiforme* are arrested in the G0/G1 phase (99.2%), whereas fungal nuclei from mycorrhizal roots are in the synthetic (S) (10.1%) and G2/M phase (3.9%). Nuclei undergoing DNA synthesis were detected in situ after BrdU uptake. Labelled nuclei were observed in intercellular hyphae and in large arbuscular trunks. This paper demonstrates that colonization of an arbuscular mycorrhizal fungus is linked to activation of its cell cycle.

Keywords: Flow cytometry; Propidium iodide; Cell cycle analysis; Bromodeoxyuridine; DNA synthesis; Immunocytochemistry; *Glomus versiforme*.

Abbreviations: AM-fungi arbuscular mycorrhizal fungi; BrdU 5-bromo-2′-deoxyuridine; PI propidium iodide; DAPI 4′,6-diamidino-2-phenylindole.

Introduction

In mutualistic symbioses, two or more partners live together exhibiting a broad spectrum of situations, ranging from those in which a host exploits its symbiont, to those in which a symbiont simultaneously exploits its host (Smith 1993). An important requisite for success is the synchronized growth and balanced co-development of the partners (Hill 1989). Without this, the symbionts would not remain together as a functional unit. Control mechanisms, probably exerted by one symbiont over the other, keep the system in equilibrium, even under variable environmental conditions (Hill 1989). One of the most widespread types of symbiosis is the arbuscular mycorrhiza, established between arbuscular mycorrhizal (AM) fungi and roots of about 80% of land plants (Harley 1989).

AM fungi are zygomycetes, whose origin 353–452 Ma ago suggests that they were instrumental in the colonization of land by ancient plants (Simon et al. 1993). The close relationship of AM fungi with their host is reflected in their obligate biotrophic status; their growth is limited in the absence of the association. Early hyphal growth depends on storage molecules in the asexual spores, which are long-term, resistant propagules, whereas symbiotic growth is exclusively root-dependent (Bécard and Fortin 1988, Bonfante and Perotto 1992). Therefore, completion of the life cycle of AM fungi depends on their ability to colonize a host plant by developing an intraradical hyphal phase, consisting of coils, intercellular hyphae, vesicles and arbuscules (Bonfante 1984).

The limited data on mechanisms underlying the development and particularly the cell cycle of AM fungi mostly refer to the occurrence of nuclear divisions in germinating spores (Bianciotto and Bonfante 1992, 1993; Bécard and Pfeffer 1993). This is mainly because of experimental difficulties in the analysis of complex, inaccessible hyphae, situated within the host, which do not grow in axenic culture.

This paper investigates the major cell cycle events in an AM fungus *Glomus versiforme* during its symbiot-

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ic phase inside the roots of *Allium porrum*. Its developmental phase is compared with the sporal resting phase. Two questions were posed: (*i*) are there changes in the ploidy level of the nuclei along the fungal hyphae? and (*ii*) are there specific fungal structures in which DNA replication occurs? Two experimental approaches were used: flow cytometry and bromodeoxyuridine (BrdU) pulse labelling. The first technique has become a powerful tool for the study of ploidy and cell-cycle parameters of mammalian cells (Darzynkiewic and Chrissman 1991, Haugland 1993) and plant cell nuclei (Galbraith et al. 1983, De Rocher et al. 1990), but presents difficulties for the analysis of microbial populations (Fouchet et al. 1993), because of the enormous variability in their cell shape and volume, and in DNA, RNA, and protein content. By contrast BrdU, a thymidine analogue, reveals replicating nuclei when used in conjunction with a specific monoclonal antibody (Stroobants et al. 1990, Oinuma et al. 1992).

A method to obtain clean nuclear suspensions from dormant spores and symbiotic mycelia and instrument optimization are also described. They were employed to analyse the G0/G1, S and G2/M phases by flow cytometry, and then to detect replicating nuclei in situ after DNA uptake of BrdU.

Materials and methods

Fungus and plant material

Seeds of *Allium porrum* L. cv. "Mostruoso di Carentan" (Sementi Dotto, Mortegliano, Udine, Italy) were sown in pots of sterilized quartz sand and watered every other day with low-phosphorus (0.75 mM $MgSO_4$, 1 mM $NaNO_3$, 1 mM K_2SO_4, 2 mM $CaCl_2$, 3.2 mM Na_2HPO_4 plus micronutrients) Long Ashton solution (Hewitt 1966). The seedlings were maintained in a growth chamber at 22 °C and 13 h light : 11 h dark regime. A spore suspension obtained from *Glomus versiforme* (Karst.) Berch sporocarps was injected around two-week-old seedlings to establish mycorrhizal infection. The degree of infection was assessed microscopically on samples stained with DAPI (4′,6-diamidino-2-phenylindole) to reveal the nuclear distribution (Bianciotto and Bonfante 1992). The percentage of total infected root lengths was evaluated by the grid-line intersect method of Giovannetti and Mosse (1980).

Isolation of nuclei and flow cytometry

Sporocarps of *G. versiforme* (0.3 g in total) were crushed in distilled sterile water to separate the spores. Spores and mycorrhizal roots of *A. porrum* were fixed in 4% p-formaldehyde (w/v) in Tris buffer (10 mM Tris (hydroxymethyl) aminomethane, 10 mM Na-EDTA, 100 mM NaCl, pH 7.4, 0.1% Triton X-100) for 10 min on ice, and then washed twice in buffer.

Nuclei were isolated by crushing the fixed spores and mycorrhizal roots with a glass rod in buffer solution (Bianciotto and Bonfante 1992). The cytoplasmic suspension was passed through a filter (cheese-cloth), and centrifuged at 1500 **g** at 4 °C. The pellet was resuspended in 1 ml saline phosphate buffer (PBS, 50 mM, pH 7.2). The suspension was passed through 15 and 5 µm pore nylon filters, centrifuged and resuspended in 0.7 ml PBS. This procedure allowed partial separation of the fungal nuclei from the plant nuclei, which were mostly retained on the filters because of their larger size (about 20 µm; Berta et al. 1990). 0.3 ml RNAse (0.5 mg/ml) was added to a 0.7 ml nuclear sample, followed by gentle mixing with 0.6 ml propidium iodide solution (PI; Sigma; 180 µg/ml in PBS). All the operations were carried out on ice (Barbiero et al. 1995).

The nuclear suspension was incubated in the dark for at least 10 min at room temperature before measuring. To check that autofluorescence did not interfere with nuclear PI fluorescence, unstained suspensions obtained both from spores and intraradical mycelia were analysed at the same time as the stained samples.

The nuclear PI fluorescence was measured with a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, U.S.A.) equipped with a 488 nm light source (argon laser). Two filters were used to collect red fluorescence from DNA-PI staining: one transmitting light at 585 nm with a band width of 42 nm (FL2), the other transmitting above 610 nm (FL3). Data were registered on logarithmic (FL3) and linear (FL2) scales. The forward scatter (FSC) and side scatter (SSC) of particles were measured simultaneously. All the data were recorded on a Hewlett Packard (HP 9000, model 300) computer, using CellFit software (Becton and Dickinson).

Debris was excluded from the analysis by raising the FL3 threshold. The appropriate threshold value was determined experimentally. Nuclear suspensions extracted from mycorrhizal *A. porrum* roots were run with the FL3 *G. versiforme* peak adjusted to channel 500 of a 1024 channel logarithmic scale. *G. versiforme* nuclei were distinguishable from debris by their low DNA red fluorescence emission (channel <200), and from the remaining *A. porrum* nuclei by their very high DNA red fluorescence emission (channel >750). Afterwards, another suspension of *G. versiforme* nuclei, extracted from spores only, was run and the area around the *G. versiforme* FL3 peak selected for DNA distribution analysis in linear mode (FL2). The corresponding red fluorescence of the *G. versiforme* G0/G1 peak in FL2 was set at channel 90.

The flow rate was set at about 200 nuclei/s, and at least 10^4 nuclei were analyzed per sample.

Experiments were repeated at least 4 times, and similar results were obtained.

Transmission electron microscopy (TEM): morphology and BrdU incorporation

Two-month-old mycorrhizal plants were fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 h at room temperature, rinsed and dehydrated in an ethanol series. The samples were then embedded in LR-White resin at 60 °C.

A two-step procedure was used to locate the replication sites. The plants were transferred into tubes containing 1 : 50 BrdU (Amersham International, Amersham, U.K.) in sterile distilled water. Preliminary experiments with different incubation times (Bianciotto and Bonfante 1993, and unpubl.) had demonstrated that 24 h treatment is needed to visualize replicating nuclei so all experiments are referred to this time. After incubation samples were rinsed twice with water for 20 min, fixed, dehydrated and embedded, as previously described. Thin sections were treated with anti-BrdU (Amersham) monoclonal antibody (MAb) supplied as a working strength solution containing nuclease for DNA denaturation. Thin sections were incu-

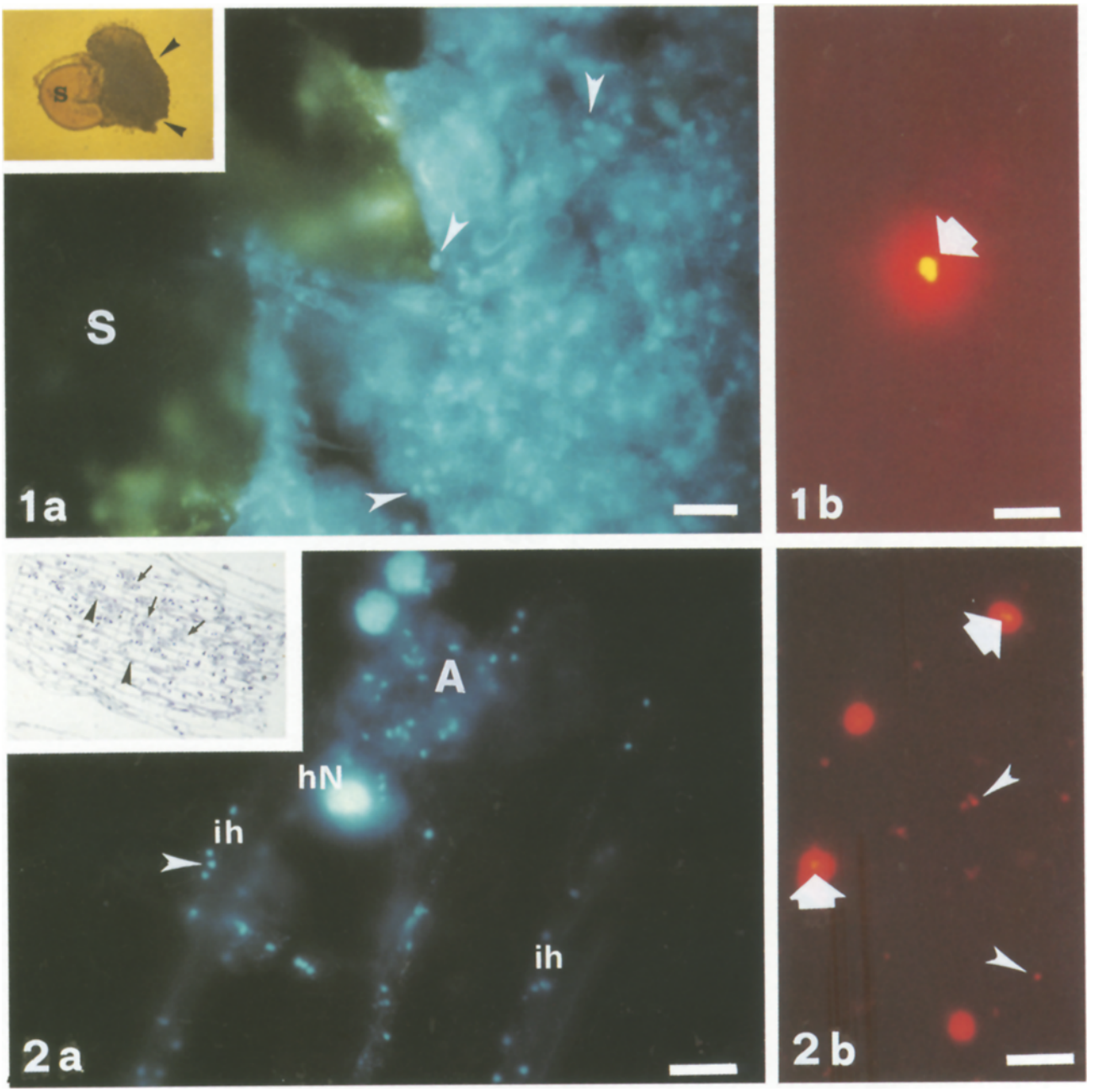
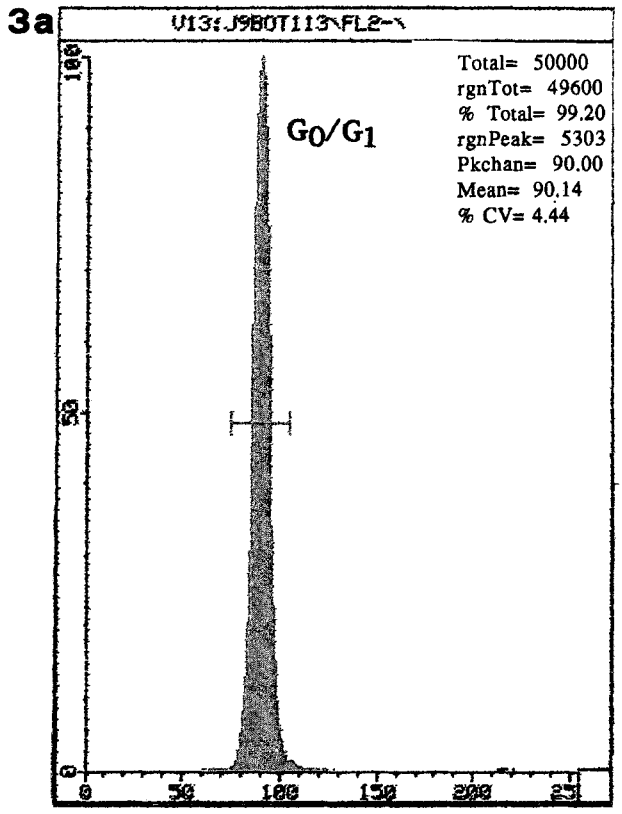
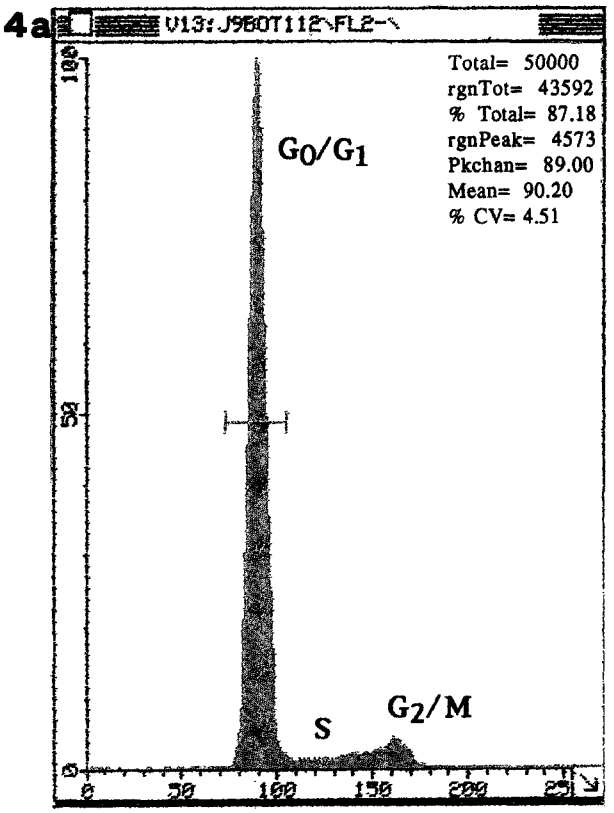


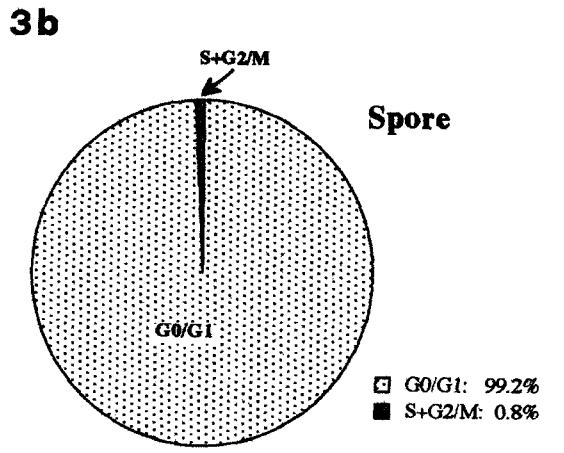
Fig. 1. a Crushed resting spores with many nuclei (about 1000) revealed by DAPI staining under UV light (arrowheads). **Inset** Crushed spore of *G. versiforme*. Note the cytoplasmic portion with many lipid bodies. Bar: 10 µm. **b** Isolated nucleus, stained with PI, extracted from spores. The clean nuclear suspension is analysed by flow cytometry. Bar: 4 µm

Fig. 2. a Frozen section of *A. porrum* infected by *G. versiforme*, showing nuclei (arrowheads) in the intercellular hyphae (*ih*) and arbuscules (*A*). The section is DAPI stained and viewed under UV light. Note the large host nuclei (*hN*). Bar: 20 µm. **Inset** Longitudinal semi-thin section of a highly mycorrhizal root stained with 1% toluidine blue. Note the numerous intercellular hyphae (arrowheads) and arbuscules (arrows). **b** Isolated fungal nuclei (arrows), stained with PI, extracted from mycorrhizal roots. Mitochondrial DNA and BLOs are also stained (arrowheads). The nuclear suspension is analysed by flow cytometry. Bar: 12 µm

bated with normal goat serum for 15 min and then exposed to undiluted MAb overnight at 4 °C. After washing with 0.05 M Tris saline buffer (TBS) pH 7.4 for 20 min and TBS containing 0.1% bovine serum albumin (BSA; Sigma) for 10 min, they were incubated with 15 nm colloidal gold-goat anti-mouse immunoglobin complex (Bio-Cell, Cardiff, U.K.) for 1 h. This second antibody was diluted 1 : 20 in 0.05 M TBS containing 1% BSA. The sections were washed with TBS, rinsed twice in distilled water and post-stained with uranyl acetate and lead citrate. Two sets of control sections were prepared: (*i*) thin sections not exposed to BrdU and (*ii*) thin sections not exposed







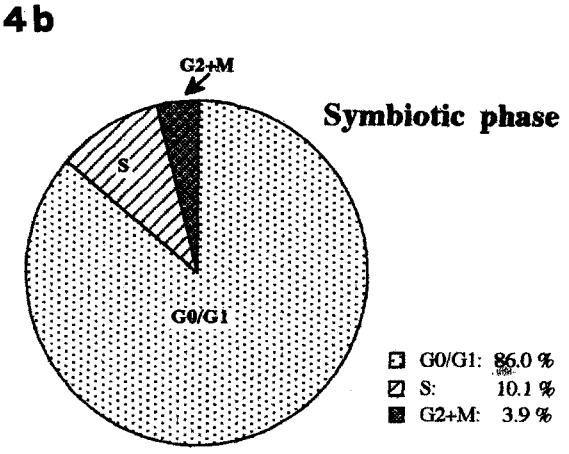


Fig. 3. a Graph of flow cytometric analysis of nuclei isolated from spores of *G. versiforme*. Fluorescence intensity of 50000 nuclei is expressed in arbitrary units (linear scale). The nuclei produce a single peak (corresponding to G0/G1 phase) at channel 90. **b** Diagram showing the distribution (%) of nuclei in G0/G1, S, G2/M cell cycle phases using a DNA-Fit SOBR or RFIT modes (see Materials and methods)

Fig. 4. a Graph of flow cytometric analysis of fungal nuclei isolated from mycorrhizal roots. Nuclei from the symbiotic mycelia form two peaks: one large peak at channel 89 (G0/G1 phase), and a smaller one at channel 165 (G2/M phase). Nuclei in synthesis (S), with an intermediate amount of DNA, are found between the two peaks. **b** Diagram showing the distribution (%) of nuclei in G0/G1, S, G2/M cell cycle phases using a DNA-Fit SOBR or RFIT modes

to MAb. These sections were also counterstained with uranyl acetate and lead citrate before examination in a Philips CM 10 TEM.

Immunogold-silver labelling for light microscopy

1 µm thick sections were placed in polylysine coated wells, treated as above, until the primary antibody treatment. They were then incubated with 5 nm colloidal gold-goat anti-mouse IgG complex. Labelling was intensified using the Intense II silver developer kit (Amersham). The sections were stained with 1% aqueous acid fuchsin and mounted in 87% glycerol.

Results

The resting spores of *G. versiforme* contain a large number of nuclei surrounded by lipid bodies, as revealed by DAPI staining (Fig. 1 a). This is typical for zygomycetes and is maintained during the symbiotic phase (Fig. 2 a), where the fungus produces inter- intracellular hyphae, coils and arbuscules (see Bonfante 1984, for a full description of the infection structures). The host nuclei are much bigger (20 µm) than those of the fungus (2 µm).

Flow cytometry

Nuclei isolated from crushed *G. versiforme* spores and mycorrhizal leek roots were labelled with PI and their purity was checked microscopically. The fungal nuclei were well-separated roundish bodies about 2 µm in diameter (Figs. 1 b and 2 b).

The spore nuclei produced one peak only at channel 90 (Fig. 3). This peak was referred to as the 1C peak, corresponding to the dominant haploid nuclear population. DNA of nuclei from the symbiotic mycelia produced two clearly separated peaks: one large peak at channel 89 and a smaller one at channel 165 (Fig. 4). The ratio between them is 1.83. Due to its overlap with the spore peak, the channel 89 peak was called the 1C level, corresponding to the presynthetic G1 phase. The other peak was named the 2C level, corresponding to the post-synthetic G2/M phase.

Nuclei in synthesis (S) with an intermediate amount of DNA were observed between the two peaks. Figures 3 and 4 show 50000 nuclei scanned by using the gated data. Distribution between the G1, S, and G2/M periods was evaluated with a DNA-fit sum of broadened rectangles (SOBR) or rectangle FIT (RFIT) modes (CellFit software, Becton and Dickinson) without differences. Almost all spore nuclei are in the G0/G1 phase (99.2%), with only 0.8% in the S–G2/M phases, whereas in the symbiotic mycelium, 86% are in the G0/G1 phase, less than 4% in the G2/M peak and 10.1% in the S phase. Percentages and S.D. from at least four experiments are shown in Table 1.

Table 1. Percentages and standard deviations (S.D.) of the G0/G1, S, G2/M cell cycle phases from at least four experiments

	G0/G1 (%, S.D.)	S (%, S.D.)	G2/M (%, S.D.)
Spore (n = 5)	97.8 ± 1.3	1.8 ± 1.1	0.4 ± 0.3
Symbiotic phase (n = 4)	90.3 ± 3.1	7.1 ± 1.6	2.6 ± 1.5

Immunolocalization

The mycorrhizal roots were examined morphologically after incubation with BrdU to reveal nuclei undergoing DNA synthesis in situ. The anti-BrdU MAb produced strong labelling of some nuclei in specific infection structures. When used with semi-thin sections the immunological reaction was revealed by silver intensification; some nuclei in the intercellular hyphae (Fig. 5) and the small penetration peg formed in cortical cells during arbuscule formation (Fig. 6) were strongly stained black. Two labelled nuclei (probably at the end of mitosis) are shown by the arrow in Fig. 5. Many nuclei were unstained in the surrounding hyphae and the thin arbuscular branches, as well as in the control experiments (Fig. 7). Those in the host root cortex were never labelled (Fig. 7) in contrast to the constant labelling of meristematic tissue.

Immunogold experiments were performed for more precise ultrastructural definition of the labelled fungal nuclei. Gold granules were evenly distributed over the non-condensed chromatin of some nuclei in the intercellular hyphae (Fig. 8), and in the large trunks of the arbuscules (Fig. 9). There were none on the nucleolus and cytoplasm (Figs. 8 and 9).

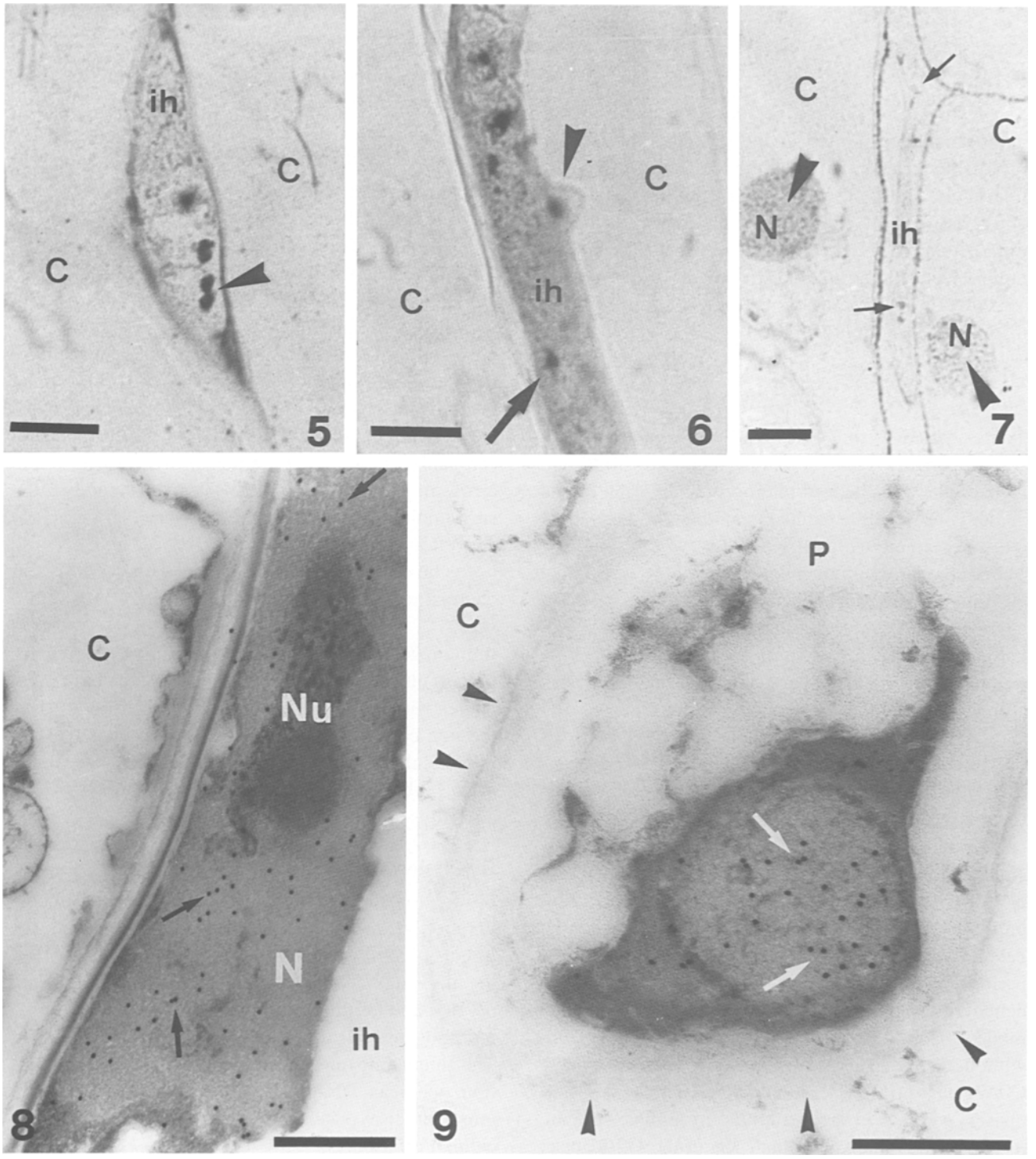
Discussion

Flow cytometry and in situ labelling after BrdU incorporation allowed us to demonstrate that (*i*) an AM fungus (*Glomus versiforme*) enters mitotic cycle during the establishment of its symbiosis with *Allium porrum* and (*ii*) the cell cycle is closely related to its morphogenesis.

The fungus moves from G0/G1 to S/M during infection, whereas DNA replication only occurs in the intercellular hyphae and the large trunks of the intracellular arbuscules.

Ploidy level, cell cycle and growth in AM fungi

AM fungi are presumed haploid, like other zygomycetes. Their genome size ranges from 0.27 pg to 1 pg per nucleus, according to species (Bianciotto and Bonfante 1992, Zézé et al. 1994, Hosny and Dulieu 1994). This ploidy level dominates their life cycle (spore, germinating hyphae, intercellular and intracellular hyphae), whereas diploidy is found in the infection structures of some pathogenic fungi, e.g., *Ustilago*



maydis (Wangemann-Budde and Schauz 1991) where a switch to the diploid phase accompanies the transition to the pathogenic phase.

Separation of the host and fungal nuclei combined with flow cytometry demonstrated that nuclei of *G. versiforme* are blocked in G0 during the sporal phase, while the cycle is activated in the presence of roots. Spores of AM fungi accumulate storage molecules (proteins, lipids and glycogen) and mobilize them during germination (Bonfante et al. 1994). As in plant embryo cells during seed germination (Georgieva et al. 1994), DNA replication and mitosis occur during the production of germinating mycelium (Bianciotto and Bonfante 1993).

The energetic costs of plant differentiation are sustained by autotrophic metabolism. Meristematic tissues can complete their cell cycle subject to several molecular controls (Francis 1992). The first control is imposed by sugar availability. Van't Hof (1973) cultured roots of various higher plants with or without sucrose and showed that cells can be arrested in G1 or G2. In the case of mycorrhizal endophytes, whose growth depends on the plant (Smith and Gianinazzi-Pearson 1988), the metabolic cost may be paid by the host. However, when spore reserves are consumed, a steady nutrient flow is prevented in the absence of the host and cell cycle is arrested and growth blocked.

In addition to metabolic support, the plant produces root exudates which support hyphal growth (Gianinazzi-Pearson et al. 1989) and under specific experimental conditions lead to the differentiation of swollen and branched hyphae (Giovannetti et al. 1993). As in plant–*Rhizobium* interactions (Peters and Verma 1990), among the components of root exudates phenolic compounds have been identified as possibly responsible for gene activation (Bécard and Piché 1992). Phenolic compounds might influence fungal divisions, supporting hyphal extension. Similar molecules activate the cell cycle in germinating pollen grains (Ylstra et al. 1992).

Activation of the fungal cell cycle was clearly indicated by flow cytometry. Even so, the percentage of G2 and mitotic events (3.9%) is rather low. *Saccharomyces cervisiae*, for example, displays rates of 19% (Dien and Srienc 1991). However, growth of an AM fungus inside a root, where the supply of nutrients is not constant, is hardly comparable with that of yeasts which are ensured a stable medium for growth. Our method may have underestimated the mitotic population by ignoring closely associated nuclei.

DNA duplication and morphogenesis in Glomus versiforme

Bromodeoxyuridine, the non-radioactive homologue of thymidine, is only taken up at specific sites in replicating fungal nuclei: i.e., intercellular hyphae

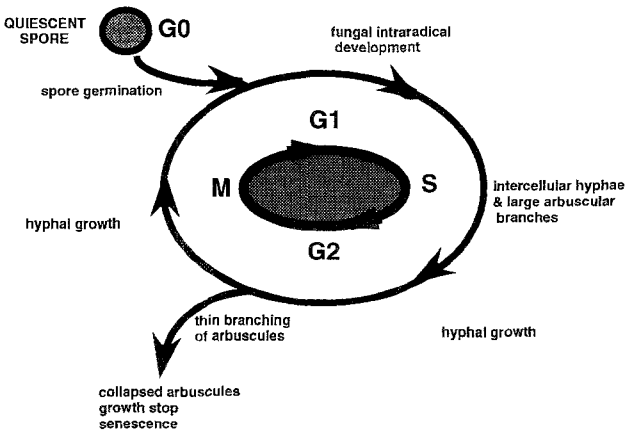


Fig. 10. The scheme presents the proposed relationship between the morphogenesis of *G. versiforme*, its cell cycle in the germinating spore and during the symbiotic phase. Nuclei within many infection structures (intercellular hyphae, large arbuscular branches) are found to be in cycle (S, G2/M). However, no DNA synthesis (S) was found in the thin arbuscular branches, suggesting that the nuclei of these specialized structures have left the cell cycle

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Figs. 5–9. Immunogold silver staining (Figs. 5–7) and immunogold labelling (Figs. 8 and 9) of semi-thin and thin sections of mycorrhizal roots of *A. porrum*, incubated for 24 h with BrdU to reveal in situ nuclei undergoing DNA synthesis. *C* Cortical cell; *ih* intercellular hypha; *N* nucleus; *Nu* nucleolus

Fig. 5. Longitudinal semi-thin section showing labelled nuclei inside an intercellular hypha. Note two labelled nuclei (arrowheads) still joined at the end of mitosis. Bar: 12 µm

Fig. 6. As Fig. 5: intercellular hypha with a labelled nucleus (arrowheads) in the penetration peg. Bar: 12 µm

Fig. 7. Longitudinal semi-thin section of a control root without MAb. No labelling occurs over the plant (arrowheads) or the fungal (arrows) nuclei. Bar: 40 µm

Fig. 8. Uniform distribution of gold granules over a nucleus in an intercellular hypha. No labelling over the nucleolus or cytoplasm. Bar: 0.4 µm

Fig. 9. Immunolocalization (arrows) of a fungal nucleus exposed to BrdU in a penetration peg (arrowheads) in cortical cells. Bar: 0.4 µm

responsible for infection diffusion in *Glomus* (Brundrett et al. 1985), and the large arbuscule trunks. Interestingly, no labelling occurred in the small arbuscule branches. Their role in the plant–fungal interaction is not fully understood. They were long regarded as the unique site for nutrient exchange, though this exclusive role (Smith et al. 1994) has recently been questioned. Intercellular hyphae may also be involved in nutritional transfer. Moreover, arbuscules only display active cytoplasm in the early stages of their formation: they are often empty, and their nuclei contain clumped chromatin (Balestrini et al. 1992). As a whole, these observations suggest that arbuscules are ephemeral structures, playing an important, if not exclusive, role in nutrient exchange, but a terminal phase in the life cycle of the fungus. The relationship between the morphogenesis of *G. versiforme* and its cell cycle during infection of the plant root are shown schematically in Fig. 10.

Experiments with BrdU performed on *G. versiforme* (this paper) and *Gigaspora margarita* (Bianciotto and Bonfante 1993) have demonstrated that DNA replication, and therefore cell cycle activation, are obligate to fungal growth. Their stability, however, depends on the presence of the host.

Interestingly the cell cycle of the root meristem is also influenced by the fungus. The plant cell cycle is slowed and metaphase longer when infection and fungal colonization proceed (Berta et al. 1990). These events occur in meristems, even though they remain uninfected. Symbiotic mycorrhizal roots – the rule in nature – can therefore be considered the result of two controls operating on the cell cycle of the symbionts: the first operates on the fungus and is closely controlled by the host; the second operates on the host and is directly or indirectly controlled by the fungus.

In conclusion, this paper demonstrates that colonization by an AM fungus requires activation of its cell cycle. This result together with the use of reliable flow cytometry provides a starting point for the investigation of molecular mechanisms underlying the regulation of a widely observed symbiosis.

Acknowledgements

The authors wish to thank Prof. S. Sgorbati and Prof. G. Berta for their advice in the early phases of the investigation, and Prof. F. Baccino for providing facilities for flow cytometry. Giuseppe Barbiero is a recipient of a fellowship of Comitato Gigi Ghirotti, Torino. The research was funded by National Research Council of Italy, Special project RAISA, Subproject no. 2, paper no. 2066.

References

Balestrini R, Bianciotto V, Bonfante P (1992) Nuclear architecture and DNA location in two VAM fungi. Mycorrhiza 1: 105–112

Barbiero G, Duranti F, Bonelli G, Amenta JS, Baccino FM (1995) Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp Cell Res 217: 410–418

Becard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri-TDNA transformed roots. New Phytol 108: 211–218

– Pfeffer PE (1993) Status of nuclear division in arbuscular mycorrhizal fungi during in vitro development. Protoplasma 174: 62–68

– Piché Y (1989) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita*. New Phytol 112: 77–83

– Douds DD, Pfeffer PE (1992) Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environ Microbiol 58: 821–825

Berta G, Sgorbati S, Soleri V, Fusconi A, Trotta A, Citterio S, Sparvoli E, Scannerini S (1990) Variations in chromatin structure in host nuclei of a vesicular arbuscular mycorrhiza. New Phytol 114: 199–205

Bianciotto V, Bonfante P (1992) Quantification of the nuclear DNA content of two arbuscular mycorrhizal fungi. Mycol Res 96: 1071–1076

– – (1993) Evidence of DNA replication in an arbuscular mycorrhizal fungus in the absence of the host plant. Protoplasma 176: 100–105

Bonfante P (1984) Anatomy and morphology of VA mycorrhizae. In: Powell CL, Bagyaraj DJ (eds) VA mycorrhizas. CRC Press, Boca Raton, pp 5–33

– Perotto S (1992) Plants and endomycorrhizal fungi: the cellular and molecular basis of their interaction. In: Verma DPS (ed) Molecular signals in plant–microbe communications. CRC Press, Boca Raton, pp 445–470

– Balestrini R, Mendgen K (1994) Storage and secretion processes in the spore of *Gigaspora margarita* Becker & Hall as revealed by high-pressure freezing and freeze substitution. New Phytol 128: 93–101

Brundrett MC, Piché Y, Peterson RL (1985) A developmental study of the early stages in vesicular mycorrhiza formation. Can J Bot 63: 184–194

Darzynkiewicz Z, Crissman HA (1991) Methods in cell biology, vol 33, flow cytometry. Academic Press, San Diego

De Rocher EJ, Harkins KR, Galbraith DW, Bohnert HJ (1990) Developmentally regulated systemic endopolyploidy in succulents with small genomes. Science 250: 99–101

Dien SB, Scrienc F (1991) Bromodeoxyuridine labelling and flow cytometric identification of replicating *Saccharomyces cerevisiae* cells: lengths of cell cycle phases and population variability at specific cell cycle positions. Biotechnol Prog 7: 291–298

Francis D (1992) The cell cycle in plant development. New Phytol 122: 1–20

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissue. Science 220: 1049–1051

Georgieva EI, López-Rodas G, Hittmair A, Feichtinger H, Broch G, Loidl P (1994) Maize embryo germination. I. Cell cycle analysis. Planta 192: 118–124

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungi by host root exudates and plant flavonoids. Symbiosis 7: 243–255

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84: 489–500

– Avio L, Sbrana C, Citernesi AS (1993) Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. New Phytol 123: 115–122

Harley JL (1989) The significance of mycorrhiza. Mycol Res 92: 92–129

Haugland RP (1993) Handbook of fluorescent probes and research chemicals, 5th edn. Molecular Probes, Eugene, OR

Hewitt EJ (1966) Sand and water culture methods used in the studies of plant nutrition. Commonwealth Agricultural Bureau, London, Tech Commun 22

Hill DJ (1989) The control of the cell cycle in microbial symbionts. New Phytol 112: 175–184

Hosny M, Dulieu H (1994) Organization of the genome of *Scutellospora castanea*, an endomycorrhizal fungus. In: Fourth European Symposium on Mycorrhizas, Granada, 11–14 July 1994, p 90

Oinuma T, Kawano J, Suganuma T (1992) Bromodeoxyuridine-immunohistochemistry on cellular differentiation and migration in the fundic gland of *Xenopus laevis* during development. Cell Tissue Res 269: 205–212

Peters NK, Verma DPS (1990) Phenolic compounds as regulators of gene expression in plant–microbe interactions. Mol Plant Microbe Interact 3: 4–8

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363: 67–68

Smith DC (1993) The symbiotic condition. Symbiosis 14: 3–15

Smith SE, Gianinazzi-Pearson V, Koide R, Cairney JWG (1994) Nutrient transport in mycorrhizas: structure, physiology and consequences for efficiency of the symbiosis. In: Robson AD, Abbott LK, Malajczuk N (eds) Management of mycorrhizas in agriculture, horticulture and forestry. Kluwer, Dodrecht, pp 103–113

– Gianinazzi-Pearson V (1988) Physiological interactions between symbionts in vesicular mycorrhizal plants. Annu Rev Plant Physiol Mol Biol 39: 221–244

Stroobants C, Sossountzoz L, Miginiac E (1990) DNA synthesis in excised tobacco leaves after bromodeoxyuridine incorporation: immunohistochemical detection in semi-thin Spurr sections. J Hist Cytol 38: 641–647

Wangemann-Budde M, Schauz K (1991) Intraspecific hybridization of *Ustilago maydis* haploids with compatible and incompatible mating type by electrofusion and genetic analysis of the fusion products. Exp Mycol 15: 159–166

Ylstra B, Touraev A, Benito Moreno RM, Stroger E, Van Tunen AJ, Vicente O, Mol JNM, Heberle-Bors E (1992) Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiol 100: 902–907

Zézé A, Dulieu H, Gianinazzi-Pearson V (1994) DNA cloning and screening of a partial genomic library from an arbuscular mycorrhizal fungus, *Scutellospora castanea*. Mycorrhiza 4: 251–254