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ORIGINAL PAPER

In situ analysis of anastomosis in representative genera of arbuscular mycorrhizal fungi

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Abstract Arbuscular mycorrhizal fungi (AMF) form obligate symbiotic associations with plants. As a result, the role of hyphal interactions in the establishment and maintenance of common mycorrhizal networks is poorly understood because of constraints on methods for *in situ* analysis. We designed a rhizohyphatron that allows the examination of intact mycelia growing from whole mycorrhizal plants. Plants preinoculated with spores were cultivated in a compartment with a connecting tube from which hyphae extend through a fine nylon mesh onto agar-coated slides. Species selected from each of the five AMF genera were used to assess and characterize the anastomosis behavior in the rhizohyphatron. Hyphal networks of *Paraglomus occultum*, *Ambispora leptoticha*, *Scutellospora heterogama*, and *Gigaspora gigantea* growing on the agar-coated slides showed no evidence of hyphal fusion. In contrast, anastomosis occurred in the hyphal networks of *Glomus clarum* and *Glomus intraradices* at an average frequency of less than 15% for both species. The rhizohyphatron developed in this study will provide knowledge of the biology and genetics of self/non-self recognition in AMF and help to better understand Glomeromycotan life history strategies.

Keywords Arbuscular mycorrhizal fungi · Glomeromycota · Anastomosis · Hyphal fusion · Vegetative compatibility

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Introduction

Fungi are modular organisms that can grow until the nutritional resources are exhausted (Andrews 1992). The continuous growth of mycelia permits fungal individuals to explore vast volumes of substrate. However, this hyphal network also poses a challenge to maintaining a continuous flow and exchange of genetic information and cytoplasmic material. A continuity of mycelia is maintained by hyphal fusion (anastomosis) that reconnects sectors that grow apart or become separated by mechanical injuries (Bago et al. 1999; de la Providencia et al. 2005). Hyphal fusions also provide a mechanism for gene flow between genetically distinct individuals within a population (Glass et al. 2000).

Anastomosis has been studied and characterized extensively in species of Ascomycete fungi (Saupe et al. 2000; Smith et al. 2000). After hyphae fuse, protoplasmic intermixing occurs in a confined sector named a heterokaryon (Pontecorvo 1956; Glass et al. 2000). The expression of vegetative incompatibility genes (*vic* genes) determines heterokaryon stability. Allelic differences between hyphae trigger the destruction of the heterokaryon and the onset of vegetative incompatibility (for reviews, see Leslie 1993; Glass and Kaneko 2003). Colonies with identical *vic* alleles are vegetatively compatible, and they maintain a stable heterokaryont mycelium capable of indefinite propagation (Glass and Dementhon 2006).

Although the mechanisms and the significance of anastomosis are well understood in Ascomycetes, understanding of hyphal fusions in populations of arbuscular mycorrhizal fungi (AMF) in Glomeromycetes is rudimentary. AMF colonize the roots of many plant species so that mycelial connectivity contributes to common mycorrhizal networks (CMNs) potentially capable of interconnecting roots of the same or different plant species (Giovannetti et

al. 2004). Little empirical evidence is available to explain the potential ecological significance of CMNs because of difficulties in making direct *in situ* observations of growing mycelia. AMF are obligate biotrophs that can only be cultured in the absence of a plant host for a brief period before a mycorrhizal association is established (Giovannetti et al. 1994, 1996). For this reason, AMF hyphal growth and development have been studied mostly in this asymbiotic phase or in monoxenic cultures of transformed roots.

Early studies reveal that different lineages of Glomeromycota exhibit diverse patterns of hyphal interactions, with unique differences in how these fungi behave during a asymbiotic versus a symbiotic phase. In the asymbiotic phase, anastomosis between germination tubes from a population of spores does not occur in *Gigaspora* and *Scutellospora* species but ranges from 34% to 90% in *Glomus* species (Giovannetti et al. 1999, 2003). In the symbiotic phase, a similar, albeit less dramatic, pattern of anastomosis is observed. The percentage of anastomosis in extraradical hyphae associated with root explants of *Gigaspora* and *Scutellospora* species is 4–10%, while anastomosis in *Glomus* species reaches 80–100% (de la Providencia et al. 2005; Voets et al. 2006). By comparison, anastomosis between the hyphae of *Glomus mosseae* growing from colonized plants is lower, ranging in frequency from 46% to 78% (Giovannetti et al. 2001, 2004). Hyphal fusions in the symbiotic phase have not been studied in any other AMF species.

Clearly, the interpretation of anastomosis behavior among external hyphae produced after the establishment of a mycorrhizal symbiosis is complicated by the experimental environment. The *in vitro* root organ culture system (Bécard and Fortin 1988) is atypical because immortalized root explants grow as autonomous organs in a medium with high soluble nutrient levels. The compatibility between fungi and the root host could be altered from the norm, as evidenced by the limited number of species that so far are maintained in culture (Declerck et al. 2005). To overcome these complications as much as possible, a cultivation system was invented in which the behavior and dynamics of hyphae generated from intact mycorrhizal plants growing in a solid substrate could be studied *in situ*. To test this cultivation system, we evaluated anastomosis within individual populations of a representative species from five glomeromycotan genera.

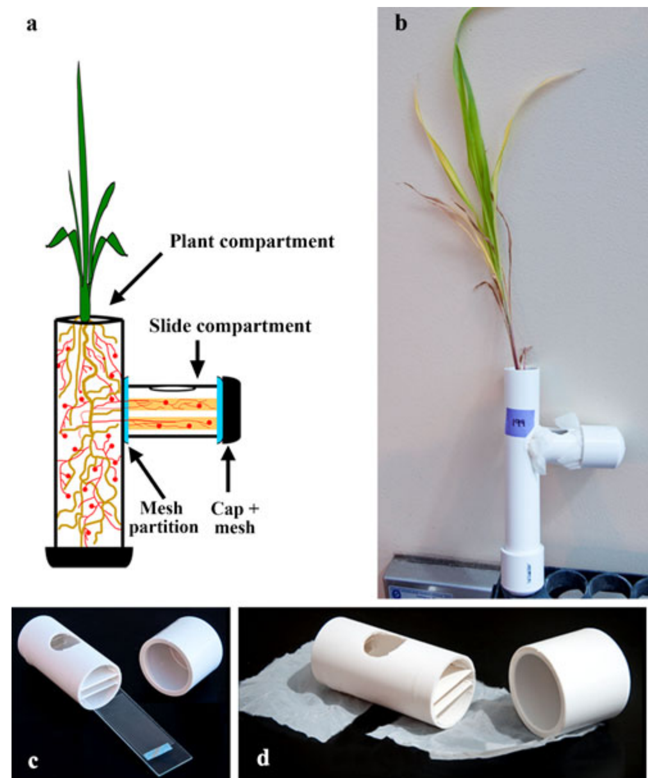

Fig. 1 Design of a rhizohyphatron used to evaluate interactions among external hyphae of arbuscular mycorrhizal fungi originating from symbiotic mycorrhizal plants. **a** Illustration of the assembled unit: a vertical plant compartment containing preinoculated mycorrhizal seedlings, a lateral slide compartment for the placement of two agar-coated glass slides, a nylon mesh with 41-μm openings so that only hyphae from the plant compartment are able to grow into the slide compartment, and a nylon mesh-and-cap combination to keep the slides in place and seal the slide compartment. **b** An assembled unit containing two 4-week-old mycorrhizal sorghum plants. **c** The slide compartment consisting of a 3.4×7-cm PVC tube with a centered 3.3-cm opening to observe and monitor hyphal growth on top slide and plastic supports for glass slides made from pot labels and affixed with 100% silicon. **d** End cap and nylon mesh with 40-μm openings to cover each end of the pictured slide compartment

Materials and methods

Rhizohyphatron and growing conditions

A “rhizohyphatron” (Fig. 1) was designed to permit the direct observation of AMF hyphae actively growing in association with roots of intact host plants growing in a solid medium. This cultivation system consisted of two compartments: (1) a plant growth compartment within which mycorrhizal plants were grown for 7 to 8 weeks and (2) a slide compartment to hold two horizontally placed agar-coated glass microscope slides that could be added, removed, or replaced at any time (Fig. 1a). The plant growth compartment consisted of a 20-cm-long PVC tube. When upright, the bottom end of the tube was sealed with a plastic cap having three small holes for water drainage. A 3.4-cm circular opening was cut approximately 8.5 cm from the top of the tube to allow the lateral insertion of the slide compartment. The slide compartment consisted of a 7.0-cm long PVC tube. Plastic supports for slides were cut from 1.0×7.5-cm plastic pot labels and attached to the wall of the

tube using 100% silicon sealant (Silicon II, General Electric Co.). The two supports in each tube were spaced 7.0 mm apart in the center of the tube (Fig. 1c). The plant and slide compartments were separated by a nylon mesh partition with 41-μm openings (Sefar America Inc., Depew, NY, USA) between the plant and slide compartments. The nylon mesh and plastic cap sealed the end of the slide chamber and provided enough resistance so that the slides were in tight contact with the nylon mesh at the juncture of the plant compartment (Fig. 1d). All plastic parts consisted of Schedule 40 PVC pipe fittings, with tubing of 3.4-cm outside diameter.

Before assembly, all components were surface-sterilized by immersion in a 10% sodium hypochlorite solution for 30 min. Plastic components were washed with distilled water, washed again in 70% ethanol, and then exposed to ultraviolet light in a laminar flow chamber for approximately 30 min.

To assemble the rhizohyphatron, a 5.0×5.0-cm nylon mesh section was held manually in place at both ends of the slide compartment, with one end inserted into the plant compartment and the other end capped and sealed (Fig. 1d). The top opening of the slide compartment was sealed with transparent tape presterilized by UV irradiation. The plant compartment was filled with Terragreen (Oil Dri Corp., Chicago, IL, USA) that had been premoistened and autoclaved by incubation at 121°C for 30 min. Two 10–12-day-old sorghum (*Sorghum sudanense* L.) seedlings were inoculated with 100–200 spores of a selected fungal isolate (Table 1) pipetted directly and evenly along intertwined roots. Before inoculation, spores were surface-cleaned by sonication and stored at 4°C for 2 days. Spores showing any signs of degradation or microbial contamination were removed. After inoculation, seedlings were transplanted into Terragreen in the plant compartment. The slide compartment was covered with a layer of aluminum foil to ensure darkness. This entire protocol was completed in a sterile laminar chamber.

Table 1 Accessions obtained from the INVAM for the *in situ* evaluation of anastomosis

Species	Accession code	Origin–contributor
Ambispora leptoticha	CR312	Costa Rica–L. Aldrich-Wolfe
Gigaspora gigantea	MN414D	Minnesota, USA–F. Pfleger
Glomus clarum	WV101	West Virginia, USA–J.B. Morton
Glomus intraradices	ON201B	Ontario, Canada–T. McGonigle
Paraglomus occultum	WY112A	Wyoming, USA–D. Watson
Scutellospora heterogama	SN722	Singapore–I. Louis

Rhizohyphatrons were placed in a growth room with a light intensity at 320 μE/m^2/s for a 14 h period and air temperature ranging from 25°C to 27°C. Plants were watered *ad libitum* daily with double-distilled deionized water and grown for 5 weeks to allow mycorrhizal development and formation of external hyphae. After 5 weeks, each rhizohyphatron was moved to a laminar flow chamber. The cap was removed from the slide compartment, two agar-coated slides were inserted, and the tube resealed with the plastic cap.

To prepare microscope slides for insertion into the rhizohyphatron, a 22×50-mm cover slip was first affixed to the center of each slide with a 3×3-mm strip of autoclave tape. A cover slip was required to observe and/or measure the details of hyphal interactions under an inverted light microscope. Slides were first autoclaved for 20 min at 121°C and then transferred to a laminar flow chamber, where they were evenly spaced in the bottom of a flat 11.5×20-cm plastic tray, with 14 slides per tray. The slides were covered with a thin, uniform layer of agar by gently pouring 22 ml of autoclaved agar to cover the bottom of the plastic tray. The 1.5% water agar contained 0.1 mg kg^{-1} P (added as KH_2PO_4) and adjusted to pH 5.5. Once the agar solidified, each slide was carefully excised using a sterile dissecting blade and placed immediately into the slide compartment. After the slides were positioned, the slide compartment was capped and the rhizohyphatron transferred to the growth room.

AM fungal isolates

Globally distributed species representing five of the major genera in Glomeromycota were chosen for this study (Table 1). One productive accession of each species was selected from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM; Morton 1993). Each accession had been started initially from many spores to adequately capture genetic diversity and then a large sample of each pot culture was used in at least six successive propagation cycles to minimize any possibility of genetic drift. Given the history of these cultures, the possibility of different genetic lineages coexisting in any one of them could not be excluded.

Microscopic evaluation of hyphal contacts

The spread of AMF external hyphae from the plant growth compartment was monitored by placing the rhizohyphatron under a stereomicroscope and inspecting the top slide through the transparent tape that covered the opening in the slide compartment. Observations began 5 days after the slides were inserted and they were repeated thereafter at 3-day intervals. When a hyphal front had advanced close to

midway across the cover slip, the slide and plant compartments were gently separated from each other in a laminar flow chamber (Fig. 2a). Cover slips were detached from each slide with forceps and immediately placed under an inverted microscope (Nikon Eclipse, TE2000-S) for examination.

On less than 25% of the slides, the agar surface became contaminated with bacteria or saprophytic fungi. These units were removed and dismantled. The source of the contaminants was traced to the plant compartment but origins never were identified definitively. Other coating, such as Millipore membranes, was tested in attempts to reduce contamination. However, hyphae failed to grow from the plant compartment onto these membranes.

Hyphae present on cover slips were verified as being of AMF origin based on the structures of each species that typically formed concomitant with hyphal development, such as spores, auxiliary cells, or saccules. To facilitate the observation and identification of hyphal contacts, each cover slip was overlaid on a 1.0×1.0-mm grid. The hyphal front served as the base reference point (Fig. 2b). Hyphal contacts were evaluated in alternate columns distal to the hyphal front for a distance of 11 mm. Therefore, the columns in the grid were evaluated at 1, 3, 5, 7, 9, and 11 mm from the hyphal front (Fig. 2b). Preliminary observations had indicated that most variations in hyphal anastomosis occurred in this region (data not shown). Within each column, alternate squares were examined, totaling an area of 11 mm^2 per column. This approach was used for the isolates of *Ambispora*, *Glomus*, and *Paraglomus* species. Because hyphal contacts were much less frequent among hyphae formed by the isolates of *Gigaspora gigantea* and *Scutellospora heterogama*, all columns and all squares in each column were examined up to 11 mm from the hyphal front. One rhizohyphatron and two cover slips were evaluated per AMF isolate.

Hyphal contacts were classified as "compatible," "incompatible," or "no recognition" based on published descriptions of vegetative interactions in AMF. Compatible hyphal interactions were identified when interacting hyphae formed bridges with complete wall fusion and protoplasmic continuity (Giovannetti et al. 1999, 2001, 2004). Interactions suggestive of incompatibility were recognized when interacting hyphae formed septa, changed shape, or both (Giovannetti et al. 2003). Hyphal contacts were characterized as no recognition when hyphae physically overlapped without any morphological changes (Giovannetti et al. 1999; de la Providencia et al. 2005). The number of hyphal contacts in each category (compatible, incompatible, or no recognition) was divided by the total number of observed hyphal contacts in order to calculate relative frequencies.

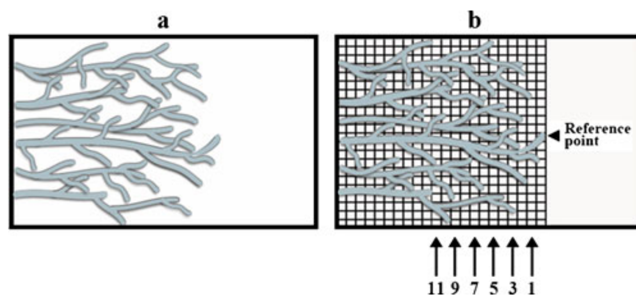

Fig. 2 **a** Illustration of AMF mycelia growing on a cover slip coated with agar. **b** cover slip overlaying a 1×1-mm grid to evaluate anastomosis. The hyphal tip most distal from the side of the cover slip nearest the plant compartment was identified as the reference point for measurements. Hyphal contacts were evaluated in alternate squares of 1-mm-wide columns (labeled *1–11*) starting at the reference edge

Results

Rhizohyphatron utility

The rhizohyphatron provided a suitable environment to conduct *in situ* observations of hyphal interactions in the symbiotic mycelium of AM fungi. With the exception of *Acaulospora*, the extraradical mycelium of all species extended into the slide compartment where the hyphal network could be examined microscopically. In most cases, the rhizohyphatron remained free of bacterial and fungal contamination for the duration of an experiment (7–8 weeks). Fungal contamination occurred in less than 25% of rhizohyphatrons and varied in both composition and abundance depending on the fungal isolate used as inoculum. Fungal contaminants always grew from the plant compartment.

Patterns of hyphal interactions in AM fungi

For all AMF species, the slide compartment was opened and cover slips were removed for microscopic analysis when the mycelia had grown an average distance of 40 mm on the agar surface. The mycelia of *Ambispora leptoticha*, *Glomus clarum*, and *Glomus intraradices* reached this distance within 7 days after the slides were placed in the rhizohyphatron. A similar distance for mycelial growth of other species was observed at 14 days. The mycelia of *A. leptoticha*, *G. clarum*, *G. intraradices* and *Paraglomus occultum* produced spores branching from older hyphae, especially near the nylon mesh partition separating the plant and slide compartments. The hyphae of *G. gigantea* and *S. heterogama* did not produce any spores, but they did form abundant auxiliary cells. Fast and bidirectional protoplasmic streaming was observed in the hyphae of *A. leptoticha*, *G. clarum*, and *G. intraradices*. In the hyphae of other species, bidirectional streaming was observed but the rate of streaming appeared to be markedly slower.

Evidence of hyphal fusion was observed only in the *Glomus* species analyzed. The hyphae of both *G. clarum* and *G. intraradices* fused by producing bridge-like structures. Bridges shorter than 50 μm were classified as "short bridges" (Fig. 3a, b), and bridges longer than 50 μm were classified as "long bridges" (Fig. 3c). Protoplasm flow between two hyphae via hyphal bridges confirmed mycelium continuity. Short and long bridges formed with similar frequency between hyphae of both species (Table 2).

A unique anastomosis behavior was observed in *G. clarum* that did not involve hyphal tips or projections. Cell wall and membrane fusion occurred between some hyphae growing in such close proximity to each other that no bridges were formed (Fig. 3d). Protoplasmic streaming was observed in regions where hyphal walls in direct contact had dissolved, thus confirming that anastomosis had occurred.

Three morphological changes in interacting hyphae were hypothesized to indicate vegetative incompatibility: (1) septation, (2) change in shape, and (3) septation accompanied by change in shape (Table 2). Septation without any change in shape occurred most frequently in the hyphae of most of the AMF species tested and it often was localized near the tips and in coarser hyphae. Septation was the only indication of vegetative incompatibility among hyphae of *G. gigantea* and *S. heterogama*. For all AMF species, mycelial compartments created by septa were devoid of cytoplasm (Fig. 4a).

Changes in the shape of the hyphae were observed in all genera except *Gigaspora* and *Scutellospora*, regardless of the occurrence of septa formation. When a hyphal tip or lateral projection contacted a putative incompatible hypha, the morphology of the former was altered (Fig. 4b). Tips became swollen with lateral expansions, and anastomosis did not occur between the interacting hyphae (Fig. 4b). This response occurred most frequently as indicative of vegetative incompatibility in the hyphae of *A. leptoticha* and *G. intraradices*. In some instances, septation occurred with a change in hyphal shape and the flow of cytoplasm became blocked (Fig. 4c).

Hyphal contacts classified as no recognition occurred most frequently among the hyphae of all AMF species studied. Hyphae established physical contact with each other, but no evidence of anastomosis, septation, or change of shape was discernible (Fig. 4d). For *P. occultum*, all hyphal interactions were characterized by no recognition.

The average frequency of compatible hyphal contacts did not exceed 15% among the two species where anastomosis occurred (Table 3), ranging from 6.7% in *G. clarum* to 13.9% in *G. intraradices*. The frequency of

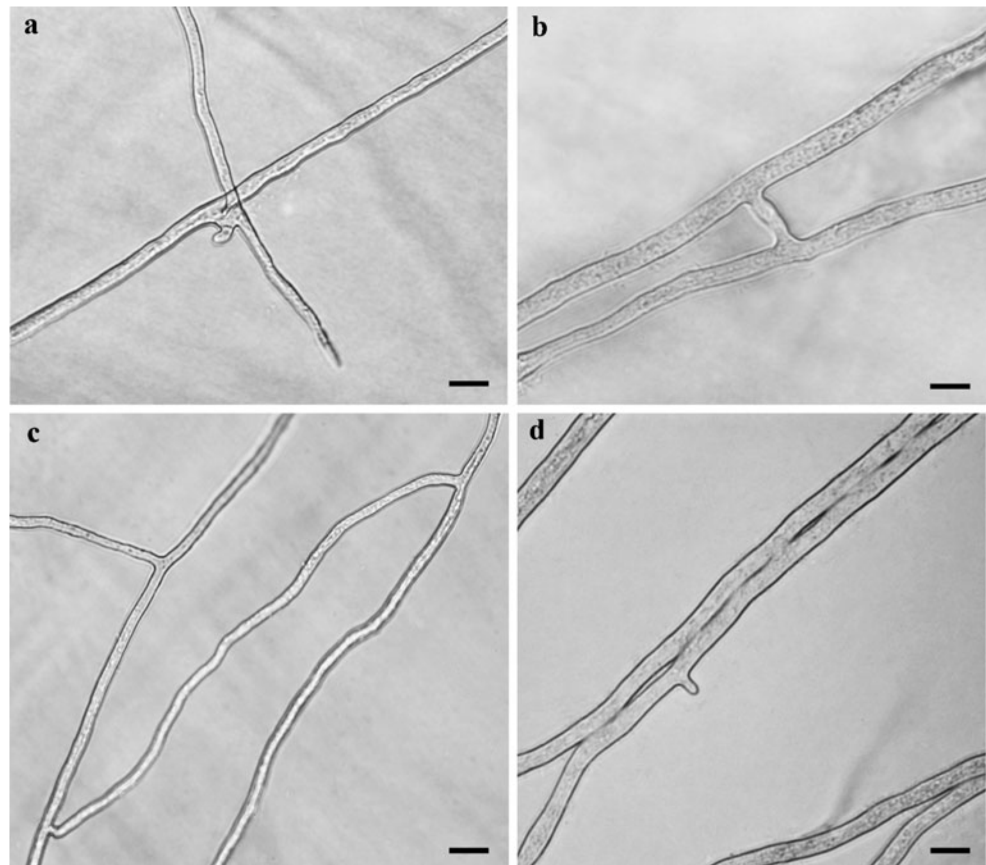

Fig. 3 Hyphal contacts in the two *Glomus* species that produced compatible hyphal fusions (anastomosis). **a**, **b** Formation of a short bridge between the hyphae of *G. intraradices* in close proximity, **c** formation of a long bridge between the more distant hyphae of *G. intraradices*, and **d** fusion of walls of *G. clarum* hyphae in direct contact with each other. *Bars*=25 μm

Table 2 Type and number of hyphal contacts indicative of compatibility and/or incompatibility of symbiotic external hyphae from the six glomeromycotan species tested, as measured on a 1×1-mm grid overlaying an agar-coated slide (see Fig. 2)

	Glomus clarum	*Glomus intraradices*	*Ambispora leptoticha*	*Gigaspora gigantea*	*Scutellospora heterogama*	*Paraglomus occultum*
Vegetative compatibility in species						
Short bridges	5	14				
Long bridges	4	12				
Parallel growth and fusion	2	0				
Total compatible contacts	11	26				
Vegetative incompatibility in species						
Septation	44	3	5	38	25	0
Change in shape	4	7	10	0	0	0
Septation + change in shape	5	4	1	0	0	0
Total incompatible contacts	53	14	16	38	25	0

incompatible contacts ranged from 7% to 32% among the six species studied. Among all AMF species, 61–100% of hyphae in physical contact with each other showed no evidence of recognition (Table 3).

Discussion

The rhizohyphatron designed for this study represents a unique tool for the *in situ* analysis of AMF external mycelia. The components of this system are easy to mass produce and maintenance is straightforward. Slides can be inserted in the rhizohyphatron at any time after the establishment of root colonization so that the mycelium can be evaluated at any stage of the symbiosis or age of the host plant.

In an open system such as this one, contamination always is the most serious concern. Less than 25% of rhizohyphatrons showed evidence of either fungal or bacterial contamination, but the absence of any pattern suggested that the most likely source was spores in the inocula. Even though spores were incubated for 2 days to permit the growth of any internal parasites, some still could have escaped detection at the time of inspection. The 2-day incubation period used in this study to detect these contaminants was probably insufficient for some spore populations. Spore germination assays and inoculation of axenic root organ cultures with a range of isolates in INVAM indicate that even the most visually clean spores may harbor internal fungal or bacterial propagules and these sometimes are not detected without a longer incubation (results not shown). In this study, the rhizohyphatrons were maintained in a room were several other pot cultures are maintained and so some contamination also from the air cannot be excluded.

The rhizohyphatron successfully allowed the anastomosis behavior in symbiotic hyphal networks to be studied for the first time in ancestral AMF species. The rhizohyphatron may also provide a useful approach in studying hyphal architecture, rates of hyphal development, and other variables *in situ*.

Anastomosis has been studied previously in fungi with membership in *Glomus* group A, *Gigaspora*, and *Scutellospora*. Our study expanded the analysis of anastomosis to include *A. leptoticha* and *P. occultum*, two species in ancestral clades (Redecker and Raab 2006). In addition to the species listed in Table 1, we also tested *Acaulospora morrowiae* (INVAM accession number WV220), but this species did not produce hyphae in the rhizohyphatron. *Acaulospora* species, in general, tend to be unpredictable even in standard pot culture environments (personal observation). Different species and growth media will need to be screened to resolve the compatibility issues in this genus.

Table 3 Total number of hyphal contacts observed in the six glomeromycotan species tested. The hyphal contacts were classified as "compatible," "incompatible," and "no recognition." The frequency of contacts (calculated as a percentage) was determined from the number of hyphal contacts in each category divided by the total number of contacts

Species	Number of contacts	Compatible contacts (%)	Incompatible contacts (%)	No recognition (%)
Ambispora leptoticha	149	0	10.74	89.26
Gigaspora gigantea	247	0	15.38	84.62
Glomus clarum	165	6.67	32.12	61.21
Glomus intraradices	187	13.90	7.49	78.61
Paraglomus occultum	118	0	0	100
Scutellospora heterogama	189	0	13.23	86.77

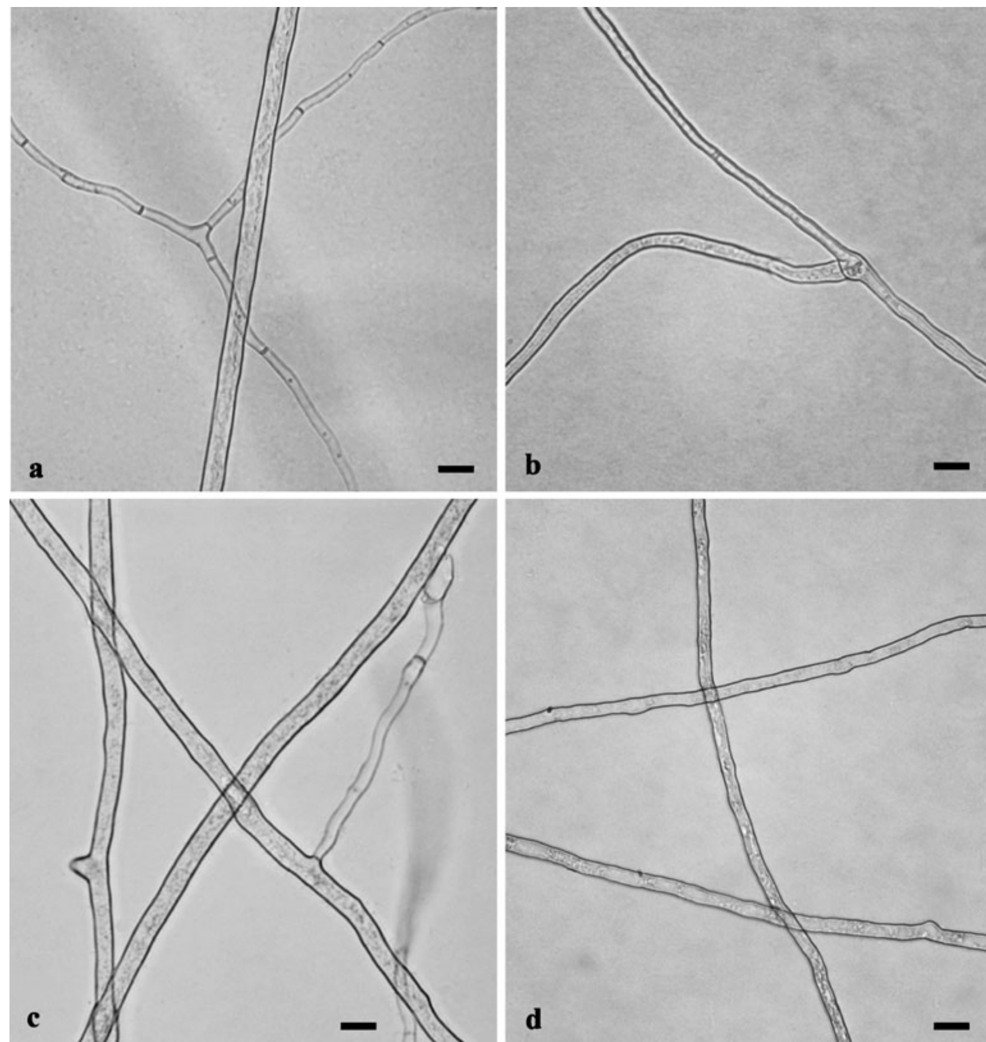

Fig. 4 Hyphal contacts suggestive of vegetative incompatibility in two AMF species. **a** Septa formation in a hypha of *G. clarum*. **b** Change in shape of a hyphal branch of *G. clarum*. **c** Septation and change in shape of a hyphal branch of *G. clarum*. **d** No recognition between the hyphae of *G. gigantea*. *Bars*= 25 μm

The frequency of hyphal fusions in *Gigaspora* and *Scutellospora* has been reported previously to average less than 10% (de la Providencia et al. 2005; Voets et al. 2006) or not to occur at all (Giovannetti et al. 1999). The results of this study support the findings of the latter study. The absence of hyphal fusion in *Archaeospora* and *Paraglomus* suggests that anastomosis is a derived trait present at least in the *Glomus* group A clade (Schüßler et al. 2001). In other fungi, anastomosis is important for colony formation to homogenize water and nutrient distribution in the mycelium and to reconstitute mycelium continuity when hyphae are injured (Gregory 1984). However, hyphal fusions are rare or absent when a population harbors deleterious cytoplasmic elements, so their transmission among individuals is avoided (Biella et al. 2002). However, without knowledge of AMF genomes, it is difficult to explain the occurrence of anastomosis in one major clade only. A similar behavior may be found in other *Glomus* clades (B and C), but those species have not yet been tested.

Evidence suggests that anastomosis in *Glomus* mediates the underground resource allocation between plants by the formation of CMNs (Mikkelsen et al. 2008) and is important for hyphae-healing mechanisms (de la Providencia et al. 2005). These benefits would be of positive value to all glomeromycotan fungi and would not be restricted to *Glomus* group A. Whatever historical events or processes involved in the emergence and heritability of anastomosis cannot be inferred from data available at this time.

The dominant type of compatible hyphal interactions observed in *Glomus* species was the formation of hyphal bridges, which has also been described in some ascomycetes. In *Neurospora crassa*, *Tuber borchii*, and *Beauveria bassiana*, for example, short bridges are produced mainly when hyphae grow parallel to each other and in close proximity (Kawamoto and Aizawa 1989; Hickey et al. 2002; Marek et al. 2003; Sbrana et al. 2007). Long bridges are observed as well in some ascomycete species, but at frequencies lower than those measured for short bridges

(Ainsworth and Rayner 1986). The occurrence of long bridges is hypothesized to involve signaling molecules required for attraction (Hickey et al. 2002). In this study, an evidence of the homing of hyphal branches towards each other before fusion supports this hypothesis. In some *Glomus* species, the existence of signaling molecules might be important because short and long bridges were formed with similar frequency.

All fusion events between vegetative hyphae reported to date involve homing of at least one hyphal tip, such as observed in the formation of bridges and "tip-to-side" fusions or pegs (Ainsworth and Rayner 1986; Aylmore and Todd 1986; Ainsworth and Rayner 1989; Correll et al. 1989; Kues 2000). The significance of the unique type of anastomosis behavior in *G. clarum*, where fusion occurred between hyphae growing parallel to each other in close proximity, is not known, but the localization of signaling molecules may intensify the opportunity for fusion events.

Both this study and the one by Giovannetti et al. (2003) indicate that any vegetative incompatibility response among symbiotic external hyphae is expressed prior to a fusion event. Given the coenocytic nature of AMF mycelium and rapid protoplasmic streaming, chemical signals that trigger vegetative incompatibility likely are expressed before anastomosis occurs. Otherwise, the spread of deleterious genetic elements across coenocytic hyphae would occur rapidly and the entire mycelium could be compromised. In contrast, signal recognition in ascomycete and basidiomycete fungi occurs during a post-fusion stage when a heterokaryont mycelium is established. In these fungi, only a small fraction of the hyphal network is compromised when there is allelic incompatibility because rapid septation would effectively block the spread of deleterious elements (McCabe et al. 1999; Marek et al. 2003).

The frequency of hyphal fusions observed in *G. clarum* was very low when compared to the results of previous studies on other species in *Glomus* group A. Anastomosis in the symbiotic mycelia of *Glomus hoi*, *G. mosseae*, and *Glomus proliferum* ranged from 44% to 100% (Giovannetti et al. 2001, 2004; de la Providencia et al. 2005; Voets et al. 2006). The type of inoculum likely was not a factor since populations of spores were used in all of these studies as well as the one reported here.

The frequency of anastomosis in *G. intraradices* was 13.9%, which is much lower than the 88% to 100% observed by de la Providencia et al. (2005) and Voets et al. (2006), respectively. However, the frequency of anastomosis could vary greatly between ecologically and geographically disjunct populations of a species, as has been observed in some basidiomycetes (Sbrana et al. 2007). Differences in cultivation systems may also influence the rate of hyphal anastomosis because of the variation in physiological processes and/or gene expression. Anastomosis in *G. intraradices* has been studied previously only in root organ cultures, which represent a selective and atypical growth environment for AMF. True comparative studies of root organ versus whole plant cultures have yet to be conducted to test the nature and magnitude of those differences.

An overall anastomosis frequency of less than 15% in glomeromycete fungi is low compared to that of fungal species in other groups. Isolates of the basidiomycete *Sclerotium rolfsii* show no evidence of incompatibility reactions (Punja and Grogan 1983). In the ascomycete *T. borchii*, the percentage of anastomosis ranges between 50 and 88% (Sbrana et al. 2007). In *Rhizoctonia solani*, anastomoses occur in more than 50% of hyphal contacts (Hyakumachi and Ui 1987).

The low frequency of anastomosis in AM fungi might be causally linked to the structure of their mycelia, which is coenocytic coupled with rapid protoplasmic streaming (personal observation; Jany and Pawlowska 2010). Although little is known about protoplasmic flow within a hyphal network, the absence of septation may allow for the continuous homogenization of cytoplasm in the different parts of a mycelium. Anastomosis then would not impact significantly on the redistribution of nutrients. Gregory (1984) reports that the coenocytic zygomycete fungi lack the ability to anastomose. However, no relationship between the inability to anastomose and mycelium structure or behavior has been established empirically.

In other fungal phyla, anastomosis allows the distribution of extra chromosomal DNA elements across a mycelium (Glass et al. 2000). A majority of studies report the frequency of hyphal fusions per hyphal length or focus on pairing isolates of different vegetative compatibility groups for the transmission of genetic elements such as viruses (Correll et al. 1989; Liu and Milgroom 1996). At the present time, it is not known if members of any glomeromycete species harbor plasmids or viruses. Anastomosis possibly plays an important role if AMF are heterokaryonts by compensating the effect of genetic drift (Pawlowska and Taylor 2004; Bever and Wang 2005), but details of the AMF genetic organization still are not decisively understood.

No sexual stage has been demonstrated in AM fungi; so, the only means for gene transfer among individuals is through the vegetative fusion of mycelia (Croll et al. 2009). Low percentages of anastomosis would suggest that the gene flow among "individuals" of AM fungi also likely is low. Other mechanisms must be active to provide genetic variability and compensate for genetic isolation.

Even though single isolate cultures were used in this study, inocula did not originate from a single spore.

Therefore, the symbiotic hyphal interactions observed in all species at present must be interpreted within the context of a population with the possibility that more than one genotype was present. Even so, the absence of anastomosis in all of the genera tested except *Glomus* group A remains enigmatic.

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