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Live-cell imaging of vegetative hyphal fusion in *Neurospora crassa*

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Abstract

The process of hyphal fusion (anastomosis) in growing colonies of *Neurospora crassa*, stained with the membrane-selective dyes FM1-43 and FM4-64, was visualized by confocal microscopy. Time-lapse, live-cell imaging illustrated the dynamics of hyphal growth and anastomosis during its pre-contact, contact and post-contact, and post-fusion stages. Fusion-competent hyphae were morphologically distinct and exhibited remote sensing, resulting in branch initiation and/or re-direction of growth to facilitate contact between participating hyphae. A stained Spitzenkörper was often observed where fusion-competent hyphae met. It is suggested that this structure contains secretory vesicles responsible for the delivery of cell adhesion molecules at the point of contact, cell wall synthesizing enzymes for the swelling growth of fused hyphal tips, and digestive enzymes required for fusion pore formation. Dramatic changes in cytoplasmic flow frequently occurred between the participating hyphae following fusion. After anastomosis has taken place, septa commonly formed close to the fusion site. The live-cell imaging reported here has clearly shown the complexity of the hyphal homing and fusion process. The control and consequences of repeated anastomoses within a mycelium must be as complex as the process itself.

1. Introduction

In filamentous ascomycetes, an individual multinucleate hypha grows by hyphal tip extension and branching (Gow, 1994; Trinci, 1984, 1994; Turner and Harris, 1997). Behind the growing colony margin, hyphae continue to branch and successively undergo fusions to yield an interconnected mycelial network that makes up the fungal individual (Fig. 1). Mycelial morphogenesis thus consists of three integrated processes: hyphal tip extension, branching, and fusion. Of these, hyphal fusion (anastomosis) is the least understood (Glass et al., 2000).

Vegetative hyphal fusion, and the capacity to form a hyphal network, has been observed in filamentous fungi since the earliest days of mycology (reviewed by Buller, 1933; Gregory, 1984). Buller (1933) was the first to outline the process of anastomosis from start to finish. However, surprisingly little has been done since that time to systematically analyze the cell biology and genetics of the formation and function of the hyphal network.

Buller (1933) provided detailed descriptions of the morphology of hyphae involved in fusions. This led to his classification system categorizing fusions based on the involvement of a hyphal tip and/or a short, sometimes barely visible, growing point termed a 'peg' (Buller, 1933, Figs. 13–16). He concluded that all fusions required a hyphal tip or peg and there was no evidence for fusions of only one tip or peg directly to the side of a hypha. It is now clear that tip-to-side fusions are not uncommon in fungi (Aylmore and Todd, 1984; Todd and Aylmore, 1985). Buller (1933) also showed that the hyphae, which become involved in anastomoses, exhibit positive autotropisms (often termed 'hyphal homing' or 'remote sensing') with tips and/or pegs growing towards each other. By contrast, the hyphal branches in the periphery of a fungal colony tend to exhibit negative autotropisms by growing away from each other (Trinci, 1984).

It is generally assumed that vegetative hyphal fusion, by networking hyphae, is important for intrahyphal communication, translocation of water and nutrients, and general homeostasis within an individual colony (Gregory, 1984; Rayner, 1996). Hyphal fusion is also

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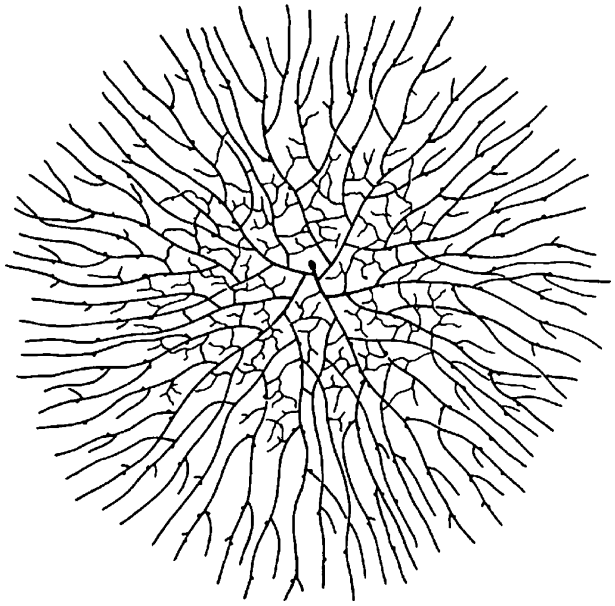

Fig. 1. Drawing showing the typical organization of a filamentous fungal colony resulting from a single germinated spore. Note the morphological differences between hyphae at the colony periphery compared with those in the interior of the colony. Anastomoses are only evident in the colony interior. Adapted from Buller (1933).

important for parasexuality and for self/non-self recognition between fungal individuals (Ainsworth and Rayner, 1986, 1989; Pontecorvo, 1956; Saupe, 2000; Worrall, 1997).

Live-cell imaging has shown that the growth, branching, and orientation of hyphae are intimately associated with the dynamic behavior of the so-called Spitzenkörper which is characteristically found within growing hyphal tips or at the sites of branch initiation (Girbardt, 1957; López-Franco and Bracker, 1996; Riquelme et al., 1998). This multicomponent structure is predominated by what are believed to be secretory vesicles (Grove and Bracker, 1970; Howard, 1981; Roberson and Fuller, 1988), which deliver enzymes, proteins, and other components to the hyphal tip where they are incorporated into the apical plasma membrane, cell wall or released into the external environment.

The overall objective of this study was to provide a detailed cytological description of the entire dynamic process of vegetative hyphal fusion in *Neurospora crassa*. This fungus was selected as a model for the analysis of anastomosis because of its fast growth rate, its tractability in culture, and the wealth of genetic and genomic information available (Davis, 2000; Davis and Perkins, 2002; Perkins et al., 2001). For this study, we have used live-cell imaging techniques with vital fluorescent dyes (FM4-64 and FM1-43) detected by confocal laser scanning microscopy (CLSM) (Fischer-Parton et al., 2000; Read and Hickey, 2001). One aim of this work was to characterize the different types of hyphal fusion in *Neurospora*. Another aim was to analyze Spitzenkörper behavior during the fusion process, since FM4-64 is an excellent stain for the apical vesicle cluster within the Spitzenkörper of growing hyphae (Fischer-Parton et al., 2000; Read and Hickey, 2001).

2. Materials and methods

2.1. Strain and culture conditions

The *N. crassa* wild-type strain 74-OR23-1VA (#2489, Fungal Genetics Stock Center, Kansas City, KS, USA) was used. Only hyphal fusions within a colony, or between two separate colonies, of this strain were imaged.

Fungi were grown on Vogel's minimal medium N (Vogel, 1956) that was modified for CLSM of hyphal fusion. Both sucrose and salts were reduced to 0.1× strength (w/v) and agar was increased to 3% (w/v) to encourage hyphae to grow on the agar surface, thus keeping them in a plane best imaged by CLSM. These methods allowed visualization of single fusion events well separated from other hyphae. Cultures were incubated prior to CLSM in darkness at temperatures ranging from 25 to 34 °C. The varying temperatures had an effect on the hyphal growth rate, but not on the fusion behavior (D.J. Jacobson, unpublished results).

2.2. Fluorescent staining

FM4-64 and FM1-43 (Molecular Probes, Eugene, OR) are membrane-selective fluorescent dyes, which differ in chemical structure and exhibit different staining patterns of fungal membranes. They are used as general cytological stains and endocytosis markers for living hyphae (Fischer-Parton et al., 2000; Read and Hickey, 2001). When added to fungal hyphae, they are incorporated into the plasma membrane, endocytosed, and then become distributed to various internal membranes, probably via the vesicle trafficking network. FM4-64 stains the Spitzenkörper well whilst FM1-43 does not. Both dyes stain the vacuolar membrane but not the nuclear envelope or cell wall.

The live-cell imaging of hyphae used an inverted agar block method (see below) with the application of dye in liquid growth medium. These dyes have limited solubility in aqueous solutions and were thus dissolved in DMSO and kept as a 16.4 mM frozen stock solution. Fresh solutions of 32 μM dye, diluted in liquid Vogel's growth medium, were prepared daily; the DMSO concentration was kept below 0.2% in the dye–medium solution added to slide cultures.

2.3. Preparation of fungi for live-cell imaging

When the mycelium had grown 3–5 cm from the point of inoculation, an agar block (ca. 1 × 2 × 0.5 cm) bearing

the colony margin was cut from the edge of the colony and inverted onto a droplet (ca. 30 μl) of liquid 0.1× strength Vogel's medium containing dye on a glass coverslip. This procedure placed the hyphae growing on the surface of the agar close against the glass for optimal imaging.

During manipulation and preparation of samples, hyphal tips often stopped growing, but resumed growth within 2–10 min. Therefore, 10 min or more of recovery time was allowed before imaging, which also gave time for dyes to be incorporated into hyphae (Fischer-Parton et al., 2000; Read and Hickey, 2001). A Petri dish lid containing a moistened paper towel was placed above the slide chamber to maintain a humid chamber while imaging over extended periods.

2.4. Differential staining of adjacent mycelia

A technique was developed for observing hyphal fusions between stained and unstained hyphae of adjacent mycelia. Agar blocks (ca. 0.5 × 2.0 × 0.5 cm) were excised from the leading edge of a *Neurospora* colony. The mycelium on the first agar block was stained with a droplet of liquid Vogel's medium containing dye and then placed mycelium-side-down on a fresh agar plate. A droplet of liquid medium without dye was applied to a similar agar block that was placed opposite the stained agar block, with a gap of 0.5–1.0 cm between the two blocks. These cultures were incubated for 2–4 h or until hyphae had grown across the gap. A new agar block was then excised from the area between the two blocks where the mycelia had grown together. This block was mounted for imaging in liquid medium without dye as described above.

2.5. Confocal microscopy

Confocal laser scanning microscopy was performed using either a Bio-Rad MRC600 or Radiance 2100 system equipped with an argon ion laser and mounted on either a Nikon Diaphot TMD or TE300 inverted microscope (all supplied by Bio-Rad Microscience, Hemel Hempstead, UK). Simultaneous, brightfield images were captured using a transmitted light detector, which collected the light from behind the microscope condenser.

FM4-64 and FM1-43 were both excited with the 514 nm laser line and their fluorescence was detected at > 550 nm. Oil immersion 60× (N.A. 1.4) or dry 20× (N.A. 0.75) plan apochromatic objective lenses were used for imaging.

The laser intensity and laser scanning of individual hyphae were kept to a minimum to reduce dye photobleaching and phototoxic effects. Time-lapse imaging was performed at scan intervals of 3–10 s for periods up to 1.5 h. Kalman filtering ($n = 2$) was sometimes used to improve the signal-to-noise ratio of individual images.

2.6. Digital image processing and animation

Images were captured using COMOS software (version 7.0, Bio-Rad) and initially viewed using Confocal Assistant software (version 4.02, freeware). Images were transferred into Paintshop Pro software (version 5.0, JASC) for further processing. Much of the analysis of time-lapse sequences involved converting them into animation movies. For this purpose, time-lapse sequences were re-sampled to convert them from .pic to .avi files to be processed by Adobe Premiere software (version 6.0). File compression was often necessary to prevent 'stuttering' playback due to the limited data transfer rate (i.e., the time taken to transfer data from hard disk to the video display hardware). Final animation movies, which accompany the figures, were produced in Quicktime format.

3. Results

3.1. Localization of hyphal fusions

Based on hyphal morphology, a *Neurospora* colony on an agar plate can be easily divided into two regions, the periphery and interior of the colony. In the colony periphery (Fig. 2A), the leading hyphae grew relatively straight and had a subapical branching pattern with primary hyphae exhibiting apical dominance over its branches. The primary hyphae and their branches usually grew in such a way that they actively avoided neighboring hyphae (negative autotropism). Nevertheless, these hyphae occasionally made contact with each other but this did not result in hyphal fusion (Fig. 2A). In these cases, two alternative behaviors were observed: (1) the growth vector of the intersecting hypha would change, usually beginning just prior to contact, and resulting in subsequent parallel growth of this hypha along the side of a resident hypha or (2) the growth vector of the intersecting hypha did not change, resulting in the contact of the hyphal tip with a resident hypha. The latter was usually followed by a short cessation of growth followed by reorientation of the tip and resumption of growth around the contacted hypha, but on a vector similar to that before contact.

The hyphal morphology of the colony interior (Fig. 2B) was distinctly different from the colony periphery (Fig. 2A). Branch initiation began at irregular intervals along the trunk hyphae laid down from growth in the peripheral zone. These interior hyphae then branched in a manner that appeared dichotomous (Fig. 2B). These branches filled the spaces between the hyphae from which they were derived. Because of this distinct branching pattern, it was difficult to assign the order (i.e., primary, secondary, and tertiary) in which branches were formed in the interior zone. It was these interior hyphae that were commonly attracted to each

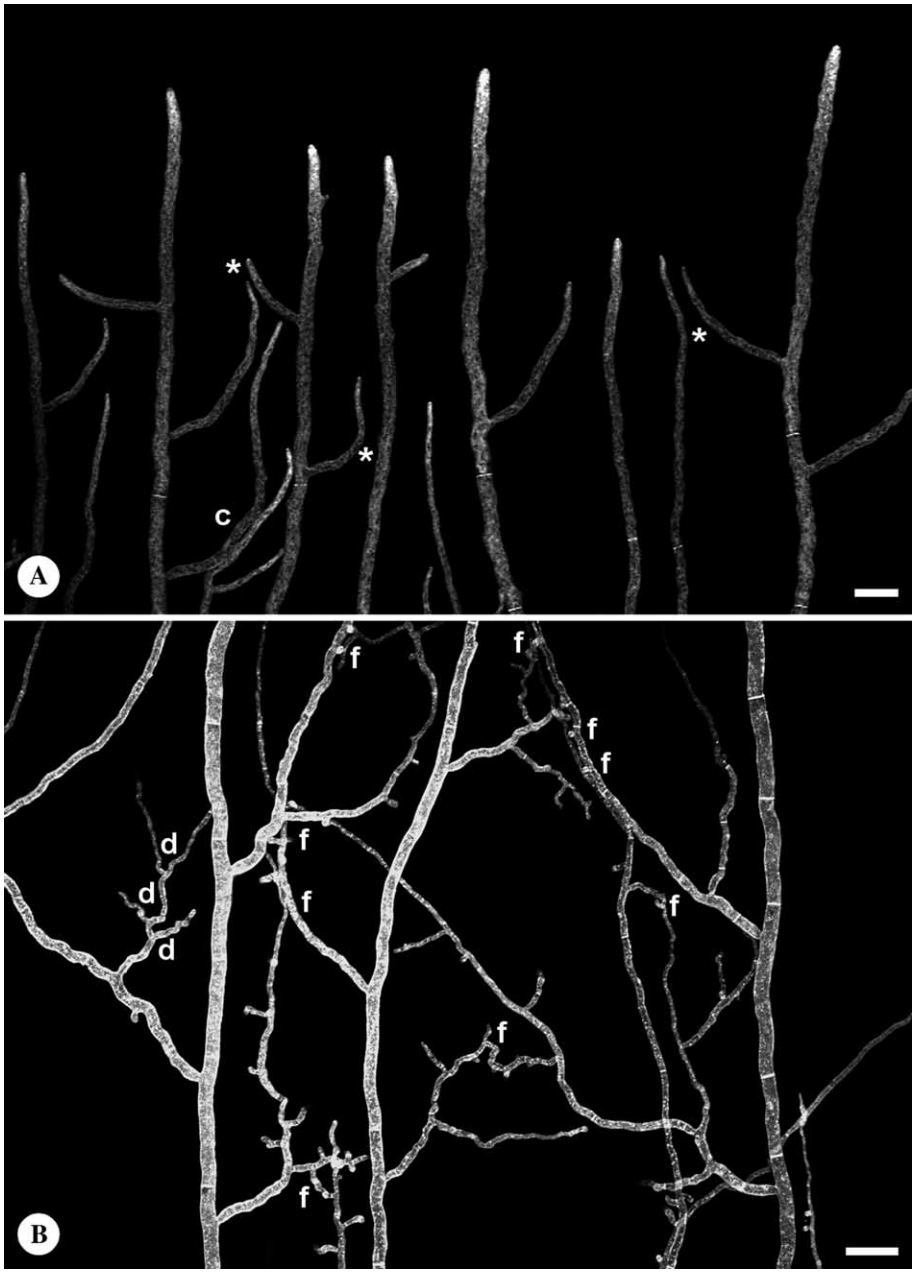

Fig. 2. Confocal images showing hyphal organization in different regions of a colony after staining with FM1-43. (A) Colony periphery (single optical section). Note the lack of hyphal fusion. Primary hyphae and branches have grown relatively straight and branched subapically. Neighboring hyphae or branches have avoided each other (e.g., in hyphal interactions indicated by asterisks). When hyphae or their branches make contact, they do not fuse with each other (e.g., see interaction indicated by c). Also see movie at www.Neurospora.org and www.sciencedirect.com from which this image was taken. (B) Colony interior (projection of six optical sections of different optical planes). Note the dichotomous branching (d) and hyphal fusions (f). The latter were determined by 3D analysis of the projection of optical sections. Bars = 50 μm.

other (positive autotropism) and anastomosed. This growth behavior gave the characteristically reticulated appearance associated with the colony interior (Figs. 1 and 2B). An additional, common morphological feature of the colony interior was short, and often multiple, fusion 'bridges' between parallel, large trunk hyphae (Figs. 3A, B). Although these trunk hyphae originated in the colony periphery, fusions between them were seen only in the interior.

The dividing line between the periphery and interior of the colony ranged from 4 to 7 mm under the growing conditions used in this study. Given that the average growth rate of hyphae at the margin of the colony in Petri dish culture grown at 34 °C is ca. 4 mm/h, hyphal fusions were first evident in the region of the colony ca. 60–105 min-old.

As colonies grew older, the density of hyphae increased to a point where visualization by CLSM of the

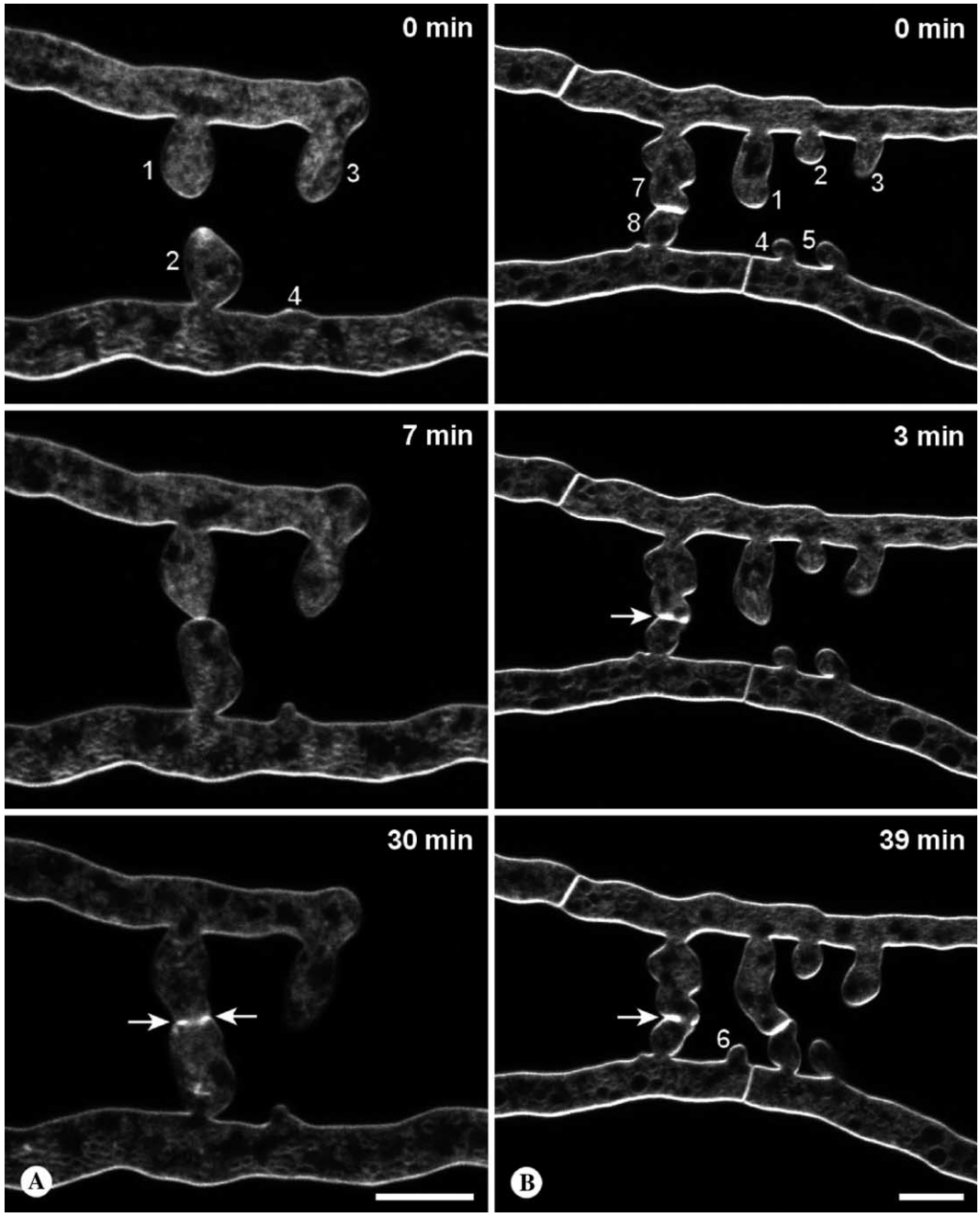

Fig. 3. Confocal images showing different stages of hyphae undergoing homing and fusion after staining with FM4-64. (A) Polarized growth of two branches (1, 2) towards each other and growth of one branch (3) towards another trunk hypha from which a peg-like branch (4) is initiated. The anastomosing branches 1 and 2 make contact (within 7 min) and form a fusion pore (within 30 min). A persistent ring of fluorescence (arrows) is present around the fusion pore after it has formed. Also see movie at www.Neurospora.org and www.sciencedirect.com from which these images were taken. (B) Multiple homing and fusion events. Note the growth of three branches (1–3) from one hypha towards two short branches on the opposite hyphae (4 and 5). After 39 min, a further branch (6) has been initiated on the lower hypha. Branches 7 and 8 fused prior to time 0. However, different stages in fusion pore (arrows) formation can be observed after 3 and 39 min. Bars = 10 μm.

interaction between individual branches was very difficult. For this reason, we limited our observations of hyphal fusions to the region where interacting branches could be individually monitored. This was roughly the transition zone between the peripheral and interior colony regions. The approximate age of the observation zone was between 70 and 160 min. In total, we observed and recorded 49 distinct fusion events. Although only six of these were observations of the complete anastomosis process from the pre-contact stage to fusion pore formation, the other 43 represented portions of the process.

3.2. Observations of the fusion process

Different morphological types of hyphal fusion were observed. Using Buller's (1933) terminology, these types were: tip-to-tip, tip-to-trunk, tip-to-peg, and peg-to-peg. However, we considered that the distinction between tips and pegs was not always clearcut and may not be of great functional significance. We therefore classified fusions as either tip-to-tip (Figs. 3A, B, 4A–C, 7) or tip-to-side (Figs. 4D, 5A, B, 6). Anastomoses were often seen in close proximity to multiple successful and unsuccessful fusion events (Figs. 3A, B, 5A).

Anastomosis involves a dynamic and continuous program of events. However, we observed distinct physiological states and transitions that hyphae apparently go through during fusion. It is convenient therefore to divide the process into the following stages: (1) pre-contact, (2) contact and post-contact, and (3) fusion pore formation and enlargement.

Pre-contact. Fusion-competent hyphae or branches were actively attracted to each other, prior to physical contact. This often involved two distinct activities: control of growth orientation (Figs. 3A, B, 4B, C) of the hyphal tips and, in some cases, initiation of new hyphal tips in response to a fusion-competent tip nearby (Figs. 3A, B, 4B, C). Both changes in growth orientation and tip initiation occurred at a distance. These observations strongly suggest that a remote-sensing mechanism, involving interhyphal signaling, is operating.

Positive autotropism, although common prior to hyphal fusion, was not always apparent. Of the 49 hyphal fusions observed, 14 (29%) involved only one tip growing toward the side wall of a trunk hypha (Figs. 4D, 5A, B, 6). In these cases, no tips were initiated and therefore there was no obvious target for the homing of hyphal tips. Only four of the 14 tip-to-side fusions were observed before contact and each growing tip contained a Spitzenkörper. However, only one of these four clearly showed a Spitzenkörper-like body in the region of the trunk hypha adjacent to the approaching tip (not shown). Whether the trunk hypha plays a role in signaling the target (point of contact) of the growing hypha could not be determined.

Contact and post-contact. Actual contact of hyphae or branches (i.e., meeting of their cell walls) could not be visualized with the methods used here due to the lack of wall staining with FM4-64 or FM1-43. Contact did not always occur at the apical poles of the tips involved (Fig. 6). Quickly after contact, however, the Spitzenkörper of participating hyphae became oriented directly opposite to each other (Fig. 4A). Hyphal extension ceased at this point and the hyphal tips became swollen (Figs. 4A, D, 5). Since the cell walls were not visible, it is unclear whether the transition from polar to non-polar growth commenced before, at, or after cell wall contact. However, the swelling of hyphal tips began while the two plasma membranes could still be visually resolved as separate from each other (not shown) and therefore prior to membrane contact. The morphology and position of the Spitzenkörper did not appear to change significantly during the polar to non-polar growth transition (not shown).

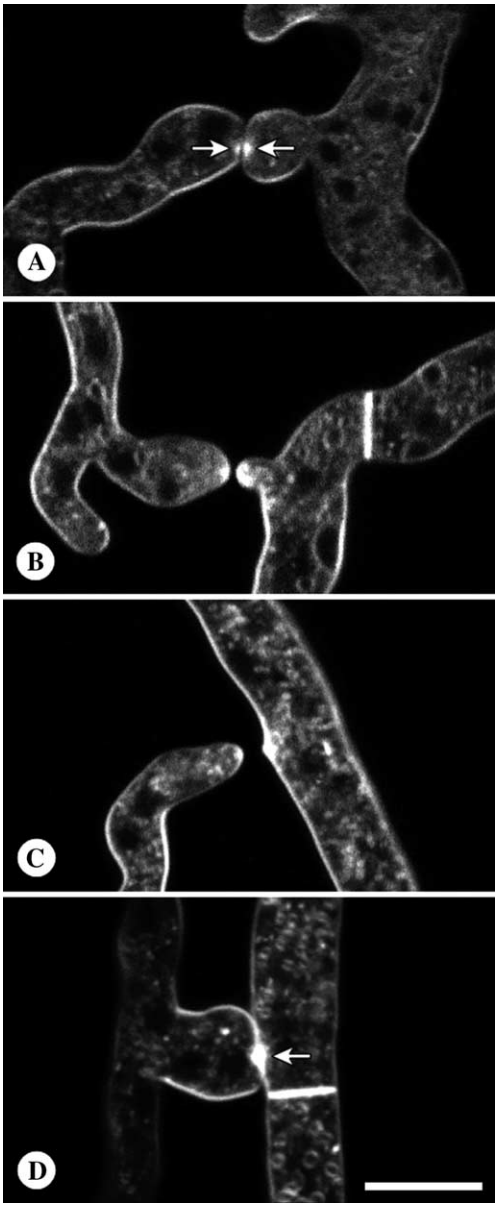

Fig. 4. Confocal images showing different examples of hyphal fusions. (A) Tip-to-tip fusion. Note Spitzenkörper (arrows) on either side of the region where pore formation will subsequently occur. (B) Two branches about to undergo tip-to-tip fusion. Note that one of the branches is very short and is peg-like. A fluorescent Spitzenkörper is present in each branch tip. (C) Two hyphal tips about to undergo fusion. Note that one of the branches has only just been initiated and is peg-like. A fluorescent Spitzenkörper is present in both the hyphal tip and the emerging peg. (D) Tip-to-side fusion. Although intense fluorescence is associated with the site of fusion (arrow) it is not clear whether one or two Spitzenkörper are present. Also see movies at www.Neurospora.org and www.sciencedirect.com. Bar = 10 μm.

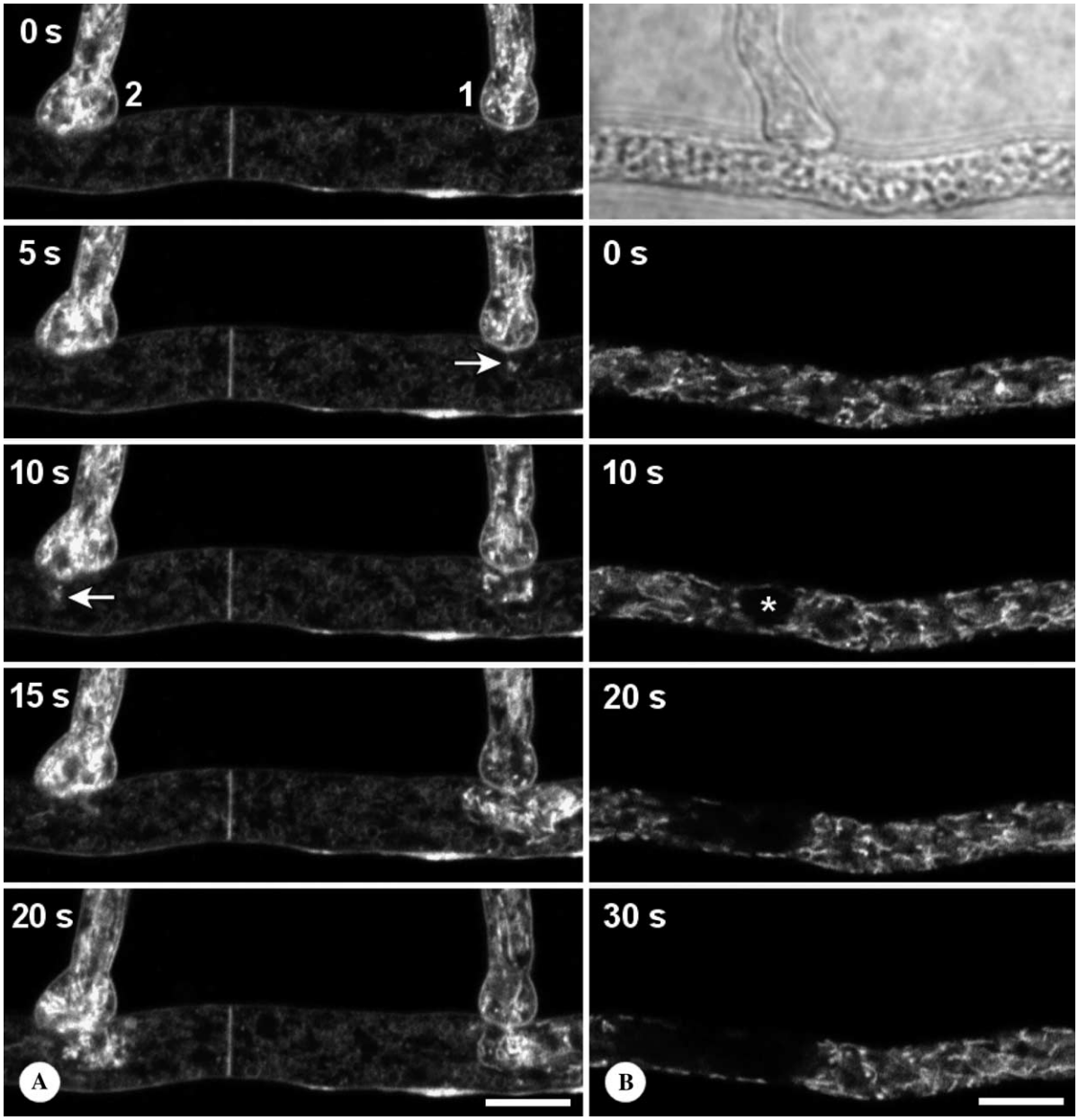

Fig. 5. Confocal images of hyphae from two separate mycelia differentially stained with FM1-43 and showing tip-to-side contact, followed by cytoplasmic flow between the fused hyphae. In each case, cytoplasm has flowed from the tips into the trunk hyphae. Note that the hyphal tips that fuse have become swollen at the point of contact. Most of the intracellular staining is of brightly fluorescent mitochondria. Also see movies at www.Neurospora.org and www.sciencedirect.com from which these images were taken. (A) The fused hyphal tips were from mycelium that was initially stained with the FM1-43 so highly fluorescent organelles can be observed to flow into the trunk hypha after cytoplasmic continuity had been initiated (at 5 s from hypha 1, arrow and at 10 s from hypha 2, arrow). The trunk hypha is lightly stained because of the transfer of a small amount of dye from the stained to the unstained agar block during the differential staining procedure (see Materials and methods). (B) The hyphal tip originated from mycelium that was not initially stained with FM1-43 so that when cytoplasmic continuity was established after 20 s an unstained region (asterisk) within the adjacent stained trunk hypha appears and then grows in size as more and more cytoplasm flows into it. Bars = 10 μm.

As the hyphal tip(s) swelled, the regions of opposing plasma membranes apparently came in contact because the two membranes could no longer be visually resolved as being separate from each other (Figs. 3A, B, 4D, 5A, 7A). Whether cell wall material was still present between the membranes could not be determined, again because stained walls were not visible. The hyphal swelling, and therefore growth, stopped prior to pore formation. The Spitzenkörper did not disappear or retract as seen in stressed hyphal tips (Girbardt, 1957; Hickey, 2001; López-Franco and Bracker, 1996), but became the point at which the fusion pore formed. Once hyphal extension had ceased, the Spitzenkörper became associated with processes involved in the fusion process (i.e., hyphal

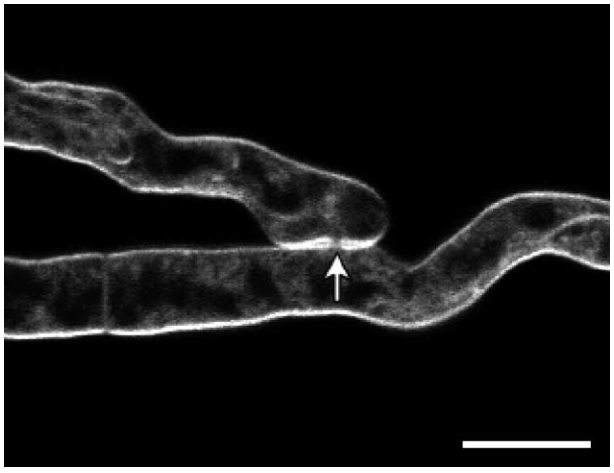

Fig. 6. Confocal image showing a tip-to-side hyphal fusion stained with FM4-64. Note that the brightly stained plasma membrane of each of the two fused hyphae is resolvable and shows apparent continuity. Also note the presence of the fusion pore (arrow). Bar = 10 μm.

adhesion, swelling, and cell wall dissolution). The Spitzenkörper during hyphal homing and fusion stained strongly with FM4-64 (Figs. 4A, D), but not with FM1-43 (Figs. 5A, B).

Fusion pore formation and enlargement. Although fusion pores could occasionally be visualized (Figs. 3A, B, 6, 7B), the only unequivocal criterion that could be used to indicate cytoplasmic continuity between two fusing hyphae or branches was when cytoplasm and organelles began flowing through the fusion pore (Figs. 5A, B). It is possible, in a few cases, that open pores were not detected because pressure differentials between fused hyphae were insufficient to cause visible flow of cytoplasm. Nevertheless, the criterion of cytoplasmic flow between fused hyphae was routinely used to indicate that fusion pores had formed with the caveat that it must be considered indirect and may be imperfect.

Often flow normally directed toward growing tips was suddenly reversed from a tip that had just fused. In some cases, subsequent flow reversed directions again within minutes of pore formation. Observing fusions between differentially stained and unstained colonies facilitated the visualization of cytoplasmic movement as a consequence of multiple hyphal fusion events (Figs. 5A, B).

The time that elapsed between apparent contact of hyphae and fusion (cytoplasmic flow) ranged from 7.5 to 17 min. During this whole period, the Spitzenkörper was present and dynamic, showing slight movement and slight changes in size and fluorescence intensity. In 81% of cases, Spitzenkörper could be observed in both hyphae after contact, on both sides of the single resolvable plasma membrane (Fig. 4A). There was never any evidence of cytoplasm leaking out of fused hyphae. After flow began, fluorescent material from the Spitzenkörper often persisted as a ring around the pore (Fig. 3A), suggesting that components of the Spitzenkörper continued to play a role in activities associated with the fusion pore.

The transition between plasma membrane integrity and cytoplasmic flow was too quick to provide any clues to the mechanism of how the pore developed. However, continuity of plasma membranes between fusing hyphae at the pore site was occasionally observed (Fig. 6). After a fusion pore formed, it sometimes enlarged slightly over a short period of time. The initial size of pores was

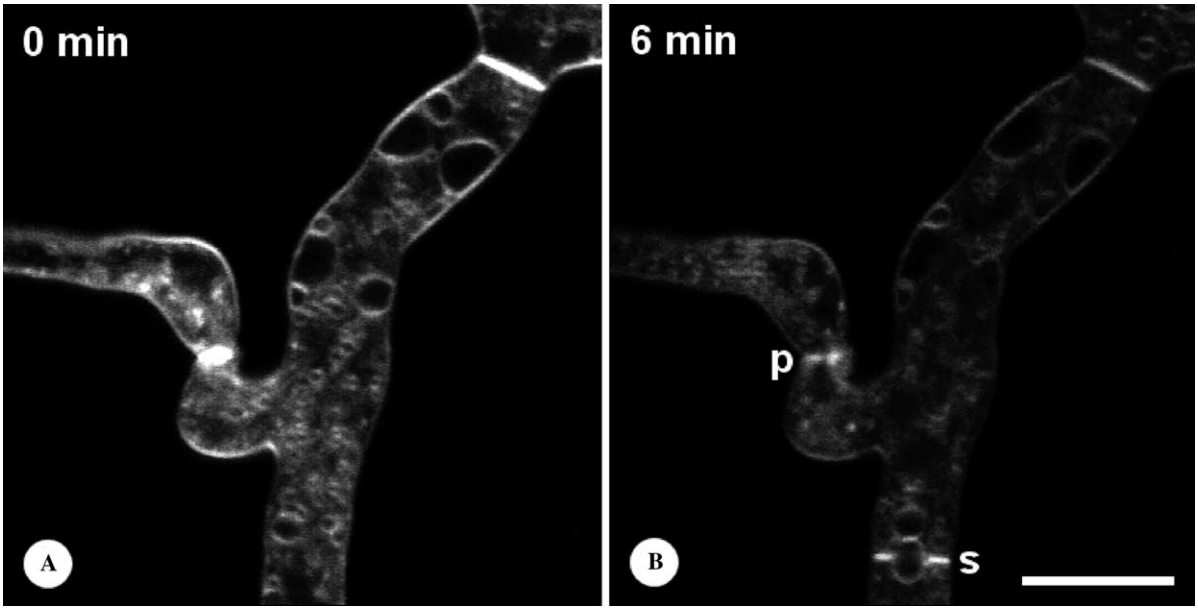

Fig. 7. Confocal image showing a tip-to-tip hyphal fusion at two different times after staining with FM-4-64 and the formation of a fusion pore (p) and a septum (s) after 6 min. Note that the dye has become photobleached during the time course series from which these two images were taken. Bar = 10 μm.

0.5–1.8 μm ($n = 40$); when they enlarged (11 out of the 40) they increased by 28–100% over the initial pore sizes.

Pore opening and the initiation of cytoplasmic flow between fused hyphae were often followed by septum formation close to the fusion site (Figs. 7A, B). Of the 49 fusion events imaged, nine fusions were correlated with septa that formed 14–61 μm from the fusion pore. Whether septa formed as a direct response to pore opening and/or cytoplasmic flow is not known.

4. Discussion

Vegetative hyphal fusion in *N. crassa*, and most likely all filamentous ascomycetes, is a highly orchestrated process apparently initiated by a physiological switch within the growing and developing mycelium that renders a portion of hyphae fusion-competent. This is followed by remote sensing of and signaling to nearby hyphae that first direct polarized growth during attraction, switch growth to a non-polarized form upon contact, and eventually stop growth altogether. Simultaneously, one or perhaps both participating hyphae coordinate the cellular machinery involved in hyphal adhesion, cell wall synthesis, and cell wall degradation resulting in the formation of a pore that connects two previously separate hyphae or branches. The end result is that tips that were growing along particular vectors are changed into conduits in which the contents from adjacent hyphal compartments are shuttled in various directions. It is a process that terminates growth of specific hyphal branches at specific locations and times during the development of a mycelium. The live-cell imaging reported here has clearly shown the complexity of the hyphal homing and fusion process. The control and consequences of repeated anastomoses within a mycelium must be as complex as the process itself.

4.1. Signaling involved in anastomosis

The most obvious criterion for whether a hypha can anastomose is its presence in the colony interior rather than periphery. The developmental switch within the colony between indeterminate exploratory growth and determinate fusion-competent growth is most likely due to a yet unknown physiologically regulated signal. The morphological consequences of this switch include apical/near apical branching, more meandering growth, positive autotropism, and, of course, anastomosis.

The pre-contact initiation of tips and redirection of hyphal growth as prerequisites for hyphal fusion are most likely due to intercellular chemical signaling between hyphae. Although the two activities of tip initiation and hyphal homing are related in time and space, they are probably different mechanistically. Tip formation is a branching event and requires de novo creation of a hyphal tip, including a Spitzenkörper (Reynaga-Pena and Bartnicki-Garcia, 1997a,b). Once the tip is formed, a different homing mechanism apparently adjusts the position of Spitzenkörper orienting growth vectors and bending the hypha to result in contact.

Clearly the growth of two hyphal tips towards each other, as a prerequisite to tip-to-tip fusion, must involve both hyphal tips signaling and responding to each other. The situation is less clear during hyphal homing leading up to tip-to-side fusions because from our observations only one in four trunk hyphae showed evidence of sensing the presence of the approaching hyphal tip by forming a Spitzenkörper in that region. Pheromones that attract hyphae to each other or between specialized structures of different genotypes are well characterized in the sexual phase of many fungi (Bistis, 1981; Kurjan, 1992; Snetselaar et al., 1996). However, intercellular signaling molecules, affecting different cells of the same genotype, may similarly control pre-contact events prior to vegetative hyphal fusion, but have so far been undiscovered.

Both fusion competency and anastomosis itself are independent of cell genotype because both self- and non-self genotypes can initiate and complete anastomosis. For *Neurospora* this includes, same or different mating types, same or different *het* genotypes, or even different species (Buller, 1933; Garnjobst and Wilson, 1956). Moreover, any interhyphal signal responsible for directing growth, initiating hyphal tips, and ultimately allowing fusion-competent hyphae to make contact must also be independent of cell genotype. This is in stark contrast to fusion events involved in fungal mating where competency to mate and mating processes are dependent on different genotypic cell types and under the control of the mating type genes (Banuett, 1998; Casselton and Kues, 1994; Coppin et al., 1997; Herskowitz, 1989). However, mechanisms controlling mating fusion events as a model for vegetative anastomosis should not be overlooked. In *Saccharomyces cerevisiae*, the process leading to mating cell fusion is initiated by cell-type specific pheromones that trigger a signal transduction pathway that ultimately results in polarization of the cytoskeleton, cell fusion, and karyogamy (Banuett, 1998; Herskowitz et al., 1995; Kurjan, 1992). Our observations suggest that polarization of the secretory apparatus, and thus presumably the cytoskeleton, is involved in hyphal fusion. In this respect, cytoplasmic microtubules have been observed in hyphal fusion bridges in ultrastructural studies of anastomoses in *Cryphonectria parasitica* (Newhouse and MacDonald, 1991).

4.2. Mechanisms of hyphal fusion

From the point of wall contact to membrane contact, two steps are apparently required: adhesion and subsequent degradation of the cell walls. The ultimate result of anastomosis is cytoplasmic continuity. We observed

considerable cytoplasmic flow through fusion pores, possibly due to different turgor pressures in fusing hyphae. No evidence of leakage of the cellular contents was observed at any fusion points, indicating that the fusing hyphae become tightly adhered to each other. Extracellular adhesive material could not be visualized using the imaging techniques employed here, although electron dense material was associated with hyphal fusion bridges in *C. parasitica*, suggesting the presence of secreted material at the hyphal fusion site (Newhouse and MacDonald, 1991).

For fusion pores to form, the fungal cell wall must be softened or degraded completely. The Spitzenkörper was invariably associated with the site of the future pore, suggesting that it might contain vesicles involved in the secretion of extracellular adhesives and cell wall degrading enzymes. Their delivery to the point of fusion may be analogous to the role that Spitzenkörper plays during hyphal tip growth, during which secretory vesicles deliver cell wall synthesizing enzymes and other components to the hyphal tip (Bartnicki-Garcia et al., 1995; Grove and Bracker, 1970; Howard, 1981; Read and Hickey, 2001). Vesicle trafficking may thus play a central role in regulating many of the processes involved in hyphal fusion. It may be important for: (1) the highly polarized delivery of wall-building vesicles for oriented growth of hyphal tips to points of fusion; (2) the less polarized delivery of wall-building vesicles to the hyphal tip so that it swells upon contact with the hypha it will fuse with; (3) the delivery of secretory vesicles containing extracellular adhesive material to stick the fusing hyphae together; and (4) the delivery of cell wall degrading enzymes to the site of fusion pore formation. It has also been argued that endocytosis may play an important role in hyphal tip growth, particularly by regulating the dynamic equilibrium in the amount of wall-synthesizing plasma membrane (Read and Hickey, 2001), which may also profoundly influence hyphal tip morphogenesis and expansion during hyphal homing, fusion, and post-contact swelling. Hyphal fusion requires that the two plasma membranes fuse, presumably in a manner similar to how vesicles and organelle membranes fuse with other membranes in eukaryotic cells (reviewed by Mayer, 2001). Even if pores are formed by physical pressure breaching the plasma membranes, the mechanism for fusing and sealing membranes must occur very rapidly.

4.3. Physiological and morphogenetic consequences of hyphal fusion

Anastomoses are thought to be a way to increase and redirect cytoplasmic flow, balancing the restricting effect of septa although, as reported by Buller (1933) and shown again here, anastomosis and septum formation are often associated. The resulting network of interconnected hyphae, and cytoplasmic flow through this network, is regulated by anastomoses (a form of 'self-plumbing') and may be important in influencing hyphal pattern formation and morphogenesis. However, post-contact consequences of hyphal fusion, involving physiological adaptation to cytoplasmic mixing and cytoplasmic flow, are virtually uncharacterized in filamentous fungi. Furthermore, the role that anastomoses play in the structure and functioning of a mycelium as a single, dynamic physiological entity, separable from post-fusion genetic interactions of self- or non-self, is often overlooked. The results presented here support the need to develop new models of fungal development at the colony level that include the influence of anastomosis. Previous conceptual paradigms focusing on hyphal tips as independent growth units become limiting when attempting to understand anastomoses and the role they play in dynamic mycelial growth and development (Davidson, 1998; Davidson et al., 1996; Rayner, 1996). This requires broader concepts, a point made quite strongly by Rayner (1996) in his discussions of 'mycelial interconnectedness.'

At present, there are no clues on what controls the frequency of, or the spatial and temporal distribution of, hyphal fusions within the fusion-competent region of the mycelium. Microenvironmental factors within the colony may play important roles in influencing this. No doubt exists, however, that hyphal fusion, as described here, is genetically controlled. Future genetic studies (combined with live-cell imaging) can be used to dissect the mechanism at each stage of the process. The complexity of the fusion process presents significant difficulties in incorporating the genetic, and perhaps epigenetic, factors involved in hyphal fusion into models of colony growth and development, and inferring from them the role of anastomoses in the life strategy of a fungus. However, the effort, although challenging, will certainly yield a more complete understanding of the fungal colony and its interaction with the environment.

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