Septum Position Is Marked at the Tip of *Aspergillus nidulans* Hyphae

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Kaminskyj, S. G. W. 2000. Septum position is marked at the tip of *Aspergillus nidulans* hyphae. *Fungal Genetics and Biology* **31,** 105–113. *Aspergillus nidulans* hyphae have long tip cells that are separated from short basal cells by septa. Basal cells average 40 μm long with three or four nuclei. Septation follows parasynchronous mitoses in the tip cell and seems to occur at premarked sites, but how these sites are established is unclear. *A. nidulans* strains with the *hypA1* mutation are wildtype at 28°C but if shifted to 42°C, their tip cells insert septa with a wildtype spacing, apparently triggered by an aberrant mitosis. Tip cell septa are trilamellar, like wildtype, but lack a central pore. Like wildtype, tip cell septation requires a minimum cell size and is inhibited by actin and microtubule poisons. In a *hypA1* background, tip cell septation is blocked by *nim* (never in mitosis) mutants, but not by *bim* (blocked in mitosis) mutants. Future septation sites appear to be established during tip growth, before their activation in basal regions.   © 2001 Academic Press

Index Descriptors: filamentous fungi; *Aspergillus nidulans;* morphogenesis; septation; *hypA.*

Choosing the right division plane is essential for daughter cell survival. Many species have uninucleate cells, whose daughter cells require a nucleus and an appropriate portion of cytoplasm. In some uninucleate fungi the division plane is related to the position of the dividing nucleus and in others it is marked by a previous division; generally the division plane is near medial. The problem is more complex in filamentous fungi.

Fungal hyphae grow by tip extension, producing multinucleate, tubular cells that divide asymmetrically by inserting cross-walls called septa. In *Aspergillus nidulans,* septation produces a long tip cell and a short basal cell (reviewed in Hamer *et al.,* 1999). Basal cells have a relatively uniform length, so the division site is not chosen randomly (Kaminskyj and Hamer, 1998; Hamer *et al.,* 1999). However, because the tip is continually extending, the cell cannot utilize markers established by previous divisions. In *A. nidulans,* synchronous mitoses in the tip cell are followed by a period of septation, and several septa can be inserted in a single round (Fiddy and Trinci, 1976). Basal cells typically contain three or four nuclei, so each mitotic nucleus does not trigger septation. Although there is good evidence that septation sites are determined prior to use (Trinci and Morris, 1979), it is unclear whether septation sites are marked by mitotic nuclei in basal regions or are created some other way during tip extension.

In fission yeasts like *Schizosaccharomyces pombe,* the future cell division plane is related to the position of the dividing nucleus, whereas in budding yeasts like *Saccharomyces cerevisiae* landmarks from the previous division determine the bud site with which both mitosis and the future division plane will be aligned. Nuclear division and cell division have been genetically dissected in yeasts, and a large number of genes involved in these processes have been identified. Despite the differences in cell division pattern, the mechanisms of cytokinesis are highly conserved between *A. nidulans* and yeasts (reviewed in Hamer *et al.,* 1999).

Wolkow *et al.* (1996) proposed that the position of the first septum is marked at germination but that this site is

not activated until the cell has grown to a threshold size, first shown by Harris *et al.* (1994). Consequently, Harris and Hamer (1995) described the first two rounds of mitosis as being "predivisional." Wolkow *et al.* (1996) also proposed that there is a tip-high inhibitor gradient that prevents septation following predivisional mitoses. In the absence of demonstrable septation site marks in apical regions, Hamer *et al.* (1999) suggested that the even spacing of subsequent septa could be explained by interactions between nuclei such that only certain mitoses could trigger septation.

Isolation of the *A. nidulans hypA1*[ts] strain has provided a new tool with which to study septum positioning. *hypA1* strains have a wildtype morphology when grown at 28°C, but at 42°C they have wide, slow-growing hyphae and delayed asexual sporulation. *hypA1* strains have a novel phenotype when colonies grown at 28°C are shifted to 42°C: the tip cells insert septa with a wildtype spacing, but later die. This is the first evidence that future septation sites may be marked in the apical regions of *A. nidulans* hyphae, even though they are not normally activated by mitotic nuclei except in basal regions.

MATERIALS AND METHODS

Strains and Growth Conditions

The strains used in this study are listed in Table 1. The *hypA1* mutant was isolated by screening a library of temperature-sensitive *A. nidulans* strains for those that were wildtype at 28°C but had nonlethal defects at 42°C including wide, slow-growing hyphae (Kaminskyj and Hamer, 1998). For most experiments, spores were grown at 28°C, in liquid complete medium (CM;[1] Kafer, 1977) amended with appropriate nutritional supplements and 50 μg/ml ampicillin (Sigma, www.sigma-aldrich.com) before temperature shift. The standard inoculum was 5 ml of 5×10^4 spores/ml in a 60-mm petri dish containing one coverslip. After 12–14 h of incubation, germlings had at least two septa. For temperature shifts, these cultures were transferred to the appropriate incubator as a single layer directly onto metal racks. For upshift, cells grown at 28°C were moved to 42°C. For upshift plus inhibitor treatment, the growth medium was replaced with 5 ml of amended medium at 28°C and shifted to 42°C. Experiments were repeated at least twice.

[1] Abbreviations used: CM, complete medium; *nim,* never in mitosis; *bim,* blocked in mitosis.

TABLE 1

Strains[a] Used in This Study

A28[b]	*biA1, pabaA6*
A777[b]	*adE20 biA1; wA2 cnxE16; sC23; methG1; nicA2; bimC4*[kinesin] *lacA1; choA1; chaA1*
A781[b]	*nimA5*[kinase]; *wA2*
ASH262[c]	*pyrG89 pabaA6 yA2; bimE7*[Apc1]
ASH80[c]	*podA1 (hypA6), pabaA6*
ASK30[d]	*hypA1; pyroA1; wA2*
ASK80[d]	*pabaA6; hypB5; chaA1*
ASK209	*hypA1; nimT23*[cdc25]; *chaA1*
ASK212	*hypA1; wA2; nicA2; bimC4*[kinesin]
ASK215	*hypA1; wA2; bimE7*[Apc1]; *pyroA4*
ASK263	*hypA1 pabaA6 yA2; nimX2*[cdc2] F233L
ASK269	*hypA1; wA2; nimX3*[cdc2] Y305H, *nicB8, pyroA4*
ASK272	*hypA1 paba A6 yA2; argB2; nimE6*[cyclinB]
ASK329	*hypA1 biA1 pabaA6; nimG10*[cyclinB]
ASK331	*hypA1 pabaA6; pyroA4; nimO18*[Dbf4]
ASK337	*hypA1 pabaA6; wA2; nimQ20*[Mcm2]
ASK343	*hypA1 pabaA6; nimP22*[polE]; *chaA1*
ASK363	*hypA1; wA2; nimA5*[kinase]
nimT[e]	*pabaA1, nimT23*[cdc25]
SO59[f]	*yA2; nimX2*[cdc2] F233L; *pyroA4; nicB8*
SO60[f]	*nimX3*[cdc2] Y305H, *nicB8, pyroA4, riboA1*
SO74[f]	*yA2; nimE6*[cyclinB]; *methG1; riboA1*
SWJ003[g]	*nimG10*[cyclinB]; *lacA1; nicB8; chaA1*
SWJ187[g]	*wA2; methB3; choA1 nimQ20*[Mcm2]
SWJ280[g]	*nimP22*[polE]; *pyroA4; chaA1*
SWJ400[g]	*pabaA1, yA2; nimO18*[Dbf4]

[a] All strains are *veA*-. Unless noted, strains were created for this study.

[b] Available from the Fungal Genetics Stock Center, University of Kansas, Kansas City, KS.

[c] From Dr. S. D. Harris, University of Connecticut Health Center, Farmington, CT.

[d] From Kaminskyj and Hamer (1998).

[e] From Dr. J. E. Hamer, Purdue University (currently, Paradigm Genetics, Research Triangle Park, NC).

[f] From Dr. S. A. Osmani, Geisinger Institute/Pennsylvania State University, Danville, PA.

[g] From Dr. S. W. James, Gettysburg College, Gettysburg, Pennsylvania.

Double mutants were constructed between *hypA1* strains and strains containing *nim* (never in mitosis) or *bim* (blocked in mitosis) mutations (Morris, 1976; Table 1) using standard techniques (Kafer, 1977). Putative double mutants were confirmed by backcrossing to wildtype.

Inhibitors

Inhibitors were prepared in liquid CM. Benomyl treatment to inhibit microtubule function used 10 μg/ml benomyl (Sigma) diluted 1000× from stock in ethanol. The control treatment for benomyl was 0.1% (v/v) ethanol. Cytochalasin A treatment to inhibit actin microfilament function used 1 μM cytochalasin A (Sigma) diluted 1000×

from stock in dimethylsulfoxide. The control treatment for cytochalasin A was 0.1% (v/v) dimethylsulfoxide. Caffeine at 1–10 mM (Sigma) was diluted from a 100 mM caffeine stock in CM. The control treatment for caffeine was CM. Inhibitor controls were not significantly different from untreated controls when average distances from the hyphal tip to the first septum were compared.

Light Microscopy

Germlings were prepared for light microscopy as described in Kaminskyj and Hamer (1998). Cells were examined with epifluorescence microscopy using a 40X, N.A. 0.75 or 63X, N.A. 1.4 objective and a Zeiss Axioplan microscope with appropriate filter sets or with a 20X, N.A. 0.50 objective and phase-contrast optics. Images were recorded on Kodak Tri-X or Kodak T-Max P3200 panchromatic film or using a Sensys CCD (Roper Scientific, www.inovis.com) driven by Metamorph imaging software (Universal Imaging, www.image1.com). Cells were measured using a calibrated ocular micrometer or with tools in Metamorph.

Cell membrane integrity was assessed by dye exclusion. Coverslips were mounted in 0.01% (w/v) aqueous toluidine blue (Sigma) containing 0.1 μg/ml ethidium bromide (Sigma; Jackson and Heath, 1990) and examined with bright-field or epifluorescence optics, respectively.

Tubulin immunofluorescence followed the method of Momany and Hamer (1997) using DM1A monoclonal IgG antitubulin (Sigma) visualized with FITC-conjugated antimouse antiserum (Sigma) and counterstained with Hoechst 33258.

Septum position was quantified as the distance from the tip to the first septum, T–S1, in the longest hypha of the germling. Statistical analysis used Statview SE + Graphics 1.02 (Abacus Concepts, www.statview.com). Graphs were prepared using Cricket Graph 1.3.1 (Cricket Software, cjs.cadmus.com/da/pages/macapps.html).

Electron Microscopy

Conidia were suspended in 0.6% (w/v) locust bean gum (Sigma) and inoculated onto sterile dialysis tubing (Spectrapor, 12,000–14,000 molecular weight cutoff) overlaid on CM agar. Growth and upshift conditions were as for petri dish cultures. All fixation and embedding steps were carried out in embryo cups with plate glass lids (Marivac, www3.ns.sympatico.ca/marivac).

Pieces of dialysis tubing with attached germlings were fixed in 1% glutaraldehyde (Marivac) in 50 mM sodium phosphate buffer, pH 7.0, for 1 h at room temperature. Samples were washed for $3\times$ 15 min in buffer, postfixed in 1% (w/v) aqueous OsO_4 in buffer for 1 h, and dehydrated in a graded ethanol series. After $2\times$ 30 min washes in dry 100% ethanol, samples were transferred through a stepwise series to dry 100% acetone, followed by $2\times$ 30 min in 100% acetone. Samples were embedded in 1:1 acetone: Epon 812 (Shell Chemical, New York, NY; no longer available) for 5 h at room temperature in a dessicator, and then the embryo cup lids were slid back slightly to allow for solvent evaporation overnight. Samples were transferred to fresh Epon on release-coated microscope slides (Electron Microscopy Sciences, www.emsdiasum.com/ems/) and covered with a second release-coated slide. The resin was polymerized at 60°C for 2 days.

Specimens were chosen using a compound microscope and phase-contrast optics, attached to Epon stubs, trimmed using a Trimtome (Gay and Faux, 1985), and sectioned on a DuPont diamond knife using a Reichert Ultracut E ultramicrotome. Silver serial sections were collected on formvar-coated copper slot grids, stained with 2% aqueous uranyl acetate and Reynold's lead citrate, and examined using a Philips CM10 or a Philips EM420 transmission electron microscope.

RESULTS

A. nidulans hyphae consist of tip cells that grow continuously and have an ongoing mitotic cycle and basal cells that are isolated from the tip by a septum(a). Basal cells are quiescent for growth and cell cycle progression until a new tip is formed for branching (Kaminskyj and Hamer, 1998). In *hypA1* strains these cell types have dramatically different responses to upshift. Tip cells stopped growing soon after upshift (cf. Figs. 1A and 1B) and inserted "tip cell septa." The basal cells swelled noticeably by 3 h at 42°C (Fig. 1B), and the swelling increased with additional time at 42°C (Fig. 1C). Tip cell septa in *hypA1* (Fig. 1D) were spaced similar to basal septa in the wildtype parent of *hypA1* (Fig. 1E).

Wildtype *A. nidulans* septum spacing is 39 $\pm$ 2 μm (SE, n = 50) when grown at 28°C (Kaminskyj and Hamer, 1998). Wildtype and *hypA1* germlings were grown for 12 h at 28°C, shifted to 42°C for 3 h, fixed, and stained with Calcofluor. Under these conditions, *hypA1* germlings produced at least three tip cell septa (Figs. 1B–1D and 2). Consequently, upshift significantly reduced the average T–S1 distance in *hypA1* germlings, from 107 $\pm$ 4 μm
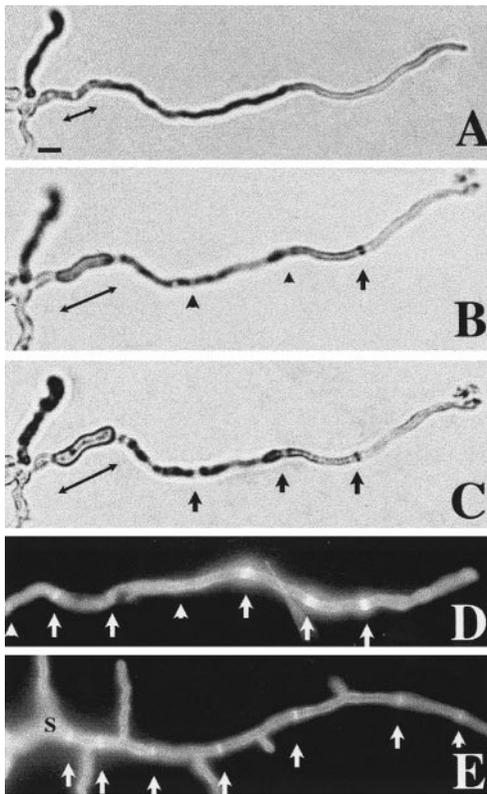
FIG. 1. Septum spacing in *Aspergillus nidulans*. (A) After 14 h at 28°C, a *hypA1* spore has germinated and produced a wildtype hyphal tip cell and a basal cell delimited by septa. (B) This germling was transferred to 42°C where, after 3 h, the tip cell had grown little but had inserted septa, and the basal cell had swollen. (C) After another 2.5 h at 42°C, the basal cell had swollen further. There are slight differences in the focal level of B and C changing the appearance of the tip cell septa. (D) A *hypA1* germling grown at 28°C for 14 h, transferred to 42°C for 1.5 h, fixed, and stained with Calcofluor to visualize wall material, has evenly spaced tip cell septa. (E) The wildtype parent of *hypA1* has evenly spaced septa in the basal regions. Arrows, septa; arrowheads, septa out of the focal plane; double-headed arrow, basal cell; S, spore. Bar, 10 μm.

before upshift to 54 ± 3 μm afterward (SE, each $n = 50$; $P = 0.001$, ANOVA). The minimum T–S1 distance recorded for an upshifted *hypA1* germling was 20 μm. In contrast to *hypA1* germlings, upshift significantly increased T–S1 distance in its wildtype parent, from 113 ± 3 μm to 148 ± 5 μm (SE, each $n = 50$; $P = 0.002$, ANOVA). Subsequent tip cell septa in *hypA1* germlings were more closely spaced than the T–S1 distance ($P = 0.03$, ANOVA), averaging 40 ± 2 μm apart (SE, $n = 156$, not including T6–7 or T7–8 shown in Fig. 2). There was no significant difference in the spacing between the subsequent tip cell septa ($P = 0.41$, ANOVA).

Timing of insertion of tip cell septa was examined in Calcofluor-stained germlings fixed at various times after upshift. The earliest time a tip cell septum was seen was 0.75 h after upshift. By 1 h, septum positions were comparable to those shown in Figs. 1D and 2. Unless stated otherwise, remaining data are for tip cells fixed 1.5 h after upshift.

Tip cell septa differed somewhat from wildtype at the ultrastructural level. Wildtype septa have a central pore associated with a Woronin body (Fig. 3A), whereas tip cell septa appeared to be complete (Fig. 3B). Examination of 18 serial sections spanning the middle third of one tip cell septum failed to reveal a pore (Fig. 3B shows a near-median section from this series) nor were pores seen in any section of a mature tip cell septum (four septa were examined). Mature tip cell septa had three layers, like wildtype, but the layers were relatively uneven. Tip cell septa formed by centripetal invagination, but this too was uneven (Fig. 3C).

The cytoplasm adjacent to developing tip cell septa was denser within ~100 nm of the cell membrane than further away (Fig. 3C). Actin has been localized by immunofluorescence to developing septa in *A. nidulans* (Harris *et al.,* 1994; Momany and Hamer, 1997) and has a cruciate array at late stages, reminiscent of this dense cytoplasmic layer (cf. Figs. 3G–3I and 4 in Momany and Hamer, 1997, and Fig. 3C in this paper). Septation in *A. nidulans* is inhibited by the actin-selective poison, cytochalasin A (Harris *et al.,* 1994; Momany and Hamer, 1997). To examine the dependence of tip cell septation on actin function, *hypA1* germ-
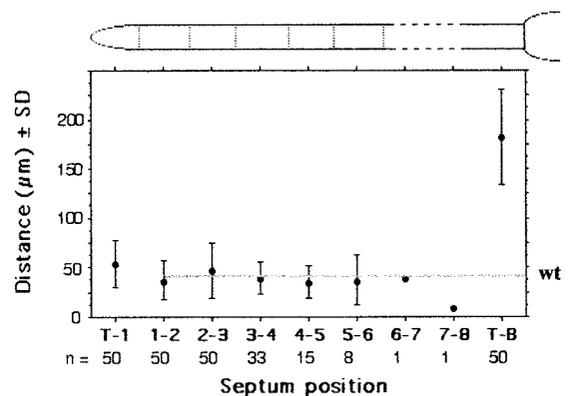

FIG. 2. Spacing of tip cell septa. *hypA1* germlings were grown at 28°C for 13 h and shifted to 42°C. After 3 h, germlings were fixed, and stained with Calcofluor to visualize cell walls. The distance between the tip and the first septum (T–S1) and between subsequent septa was measured for 50 germlings. The gray line labeled "wt" indicates the average spacing between wildtype septa (Kaminskyj and Hamer, 1998).
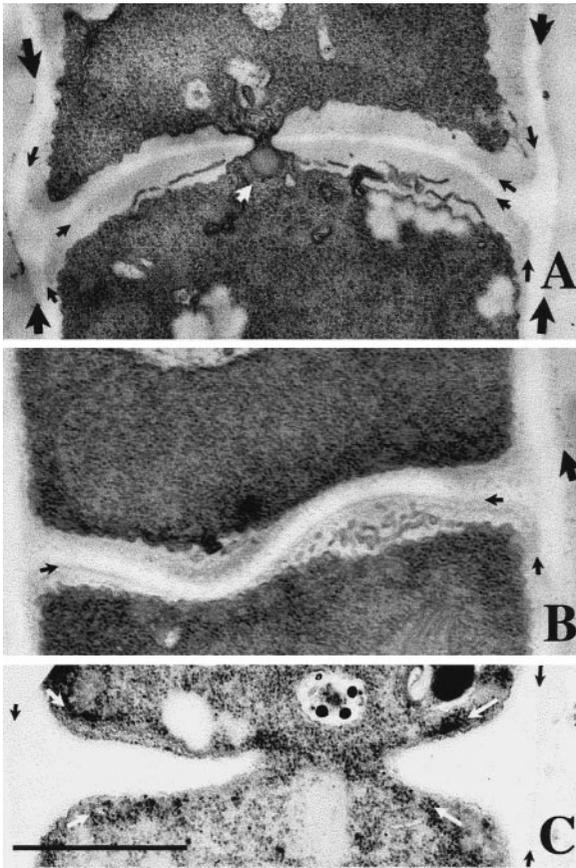
FIG. 3. Electron micrographs of near-median longitudinal sections of chemically fixed wildtype and tip cell septa. A and B are printed to highlight wall layers, which stain poorly, and C is printed to show details in the cytoplasm. (A) Wildtype septa are trilamellar (layers are indicated by black arrows) and have a central pore that can be blocked by a Woronin body (white arrow). (B) Tip cell septa have an uneven trilamellar structure (layers indicated by arrows) and lack a pore. (C) Tip cell septa formed by centripetal invagination. Black arrows indicate the outer edge of the hyphal wall. The cytoplasm adjacent to and between the edges of the forming septum is relatively dense (white arrows). Bar, 1 μm.

lings were treated with cytochalasin A and shifted to 42°C. The average T–S1 distance in cytochalasin-treated up-shifted cells was significantly greater than in control up-shifted cells ($P < 0.05$, ANOVA; Fig. 4).

A. nidulans uses mitotic nuclei as temporal cues for septation (Harris *et al.,* 1994; Wolkow *et al.,* 1996; Momany and Hamer, 1997). What happens to tip cell nuclei after upshift? *hypA1* germlings were grown for 13 h at 28°C and shifted to 42°C, and samples were taken at least every 0.5 h. In germlings fixed without upshift ~95% of the tip cells had interphase nuclei and cytoplasmic micro-

tubule arrays (Fig. 5A). By 1 h after upshift, about half of the tip cells had numerous small Hoechst 33258-staining regions cluttered throughout the cytoplasm, which were interpreted as being condensed nuclei or nuclear fragments. By 1.5 h after upshift about 80% of tip cells had these fragments. In *hypA1* germlings fixed 1 and 1.25 h after upshift, nuclei in about a quarter of tip cells colocalized with spindle-shaped tubulin arrays (Figs. 5B and 5C), suggesting that these nuclei were undergoing an aberrant mitosis. Other tip cells had the small Hoechst 33258-staining regions associated with cytoplasmic microtubules. The latter pattern became predominant at 1.5 h after upshift (Fig. 5D). If tip cell septation were triggered by this unusual mitosis, it might be inhibited by benomyl, which blocks *A. nidulans* mitosis at metaphase (Harris *et al.,* 1994). The average T–S1 distance in benomyl-treated, upshifted germlings was significantly greater than in controls ($P < 0.05$, ANOVA; Fig. 4).

Wildtype septation requires entry into and exit from mitosis, controlled by *nim* and *bim* genes, respectively (Morris, 1976). Both *nim* and *bim* mutants have an aseptate restrictive phenotype. The effect of entry into and exit from mitosis on tip cell septation was examined by constructing double mutants between *hypA1* and strains con-
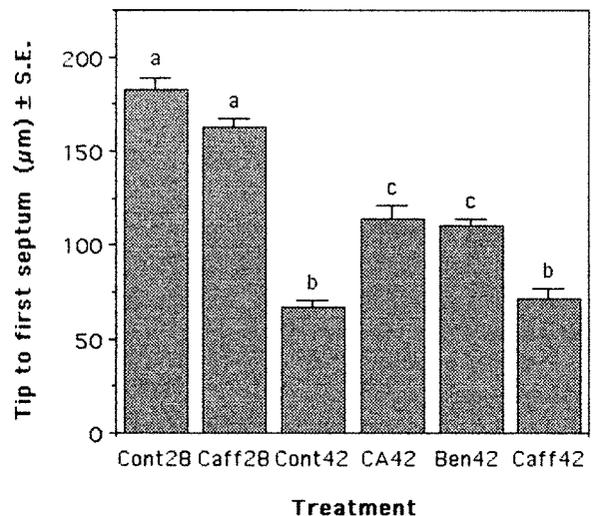

FIG. 4. Effect of inhibitors on tip-first septum distance (μm $\pm$ SE, $n = 50$). Control cells grown for 12 h at 28°C have long tip cells, unlike those shifted to 42°C for 1.5 h. Germlings treated at the time of upshift with benomyl (Ben) or cytochalasin A (CA) had significantly longer average T–S1 distances after 1.5 h at 42°C than control cells at 42°C. Caffeine (Caff) treatment did not significantly affect T–S1 distance at 28 or 42°C, compared to controls. Letters indicate significant differences at $P \leq 0.05$ (ANOVA).
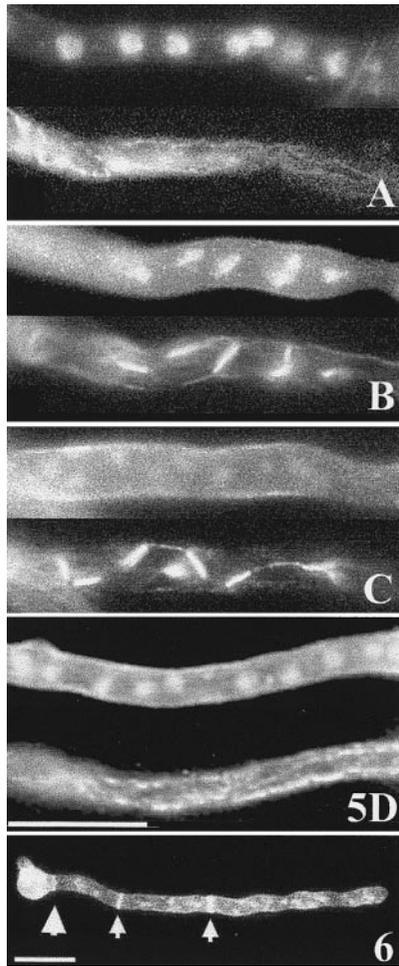
FIG. 5. Hoechst 33258 and tubulin immunofluorescence patterns in *hypA1* germlings grown at 28°C (A) and after upshift to 42°C (B–D). Each pair of images shows Hoechst 33258-stained nuclei (top) and the tubulin immunofluorescence (bottom). About 95% of tip cells before upshift (A) have interphase nuclei and cytoplasmic microtubules. Condensed nuclei in tip cells fixed 1 (B) and 1.25 h (C) after upshift had tubulin immunofluorescence patterns resembling mitotic spindles. By 1.5 h after upshift (D) most nuclear fragments were associated with cytoplasmic microtubules. Bar, 10 μm.

FIG. 6. Tip cell septation in a *hypA1* germling grown at 28°C for 8.5 h and then shifted to 42°C for 1.5 h. Under these conditions germlings inserted as many as four septa although growth was limited (Table 2). Large arrowhead shows the position of the only septum expected in a germling this size. Arrows show tip cell septa. Bar, 10 μm.

taining temperature-sensitive mutations in *nim* and *bim* genes. These were *nimA*[kinase] (Osmani *et al.,* 1991), *nimE*[cyclinB] (O'Connell *et al.,* 1992), *nimG*[cyclinB] (S. Osmani, personal communication), *nimO*[Dbf4] (James *et al.,* 1999), *nimP*[polE] (S. W. James, personal communication), *nimQ*[Mcm2] (Ye *et al.,* 1997), *nimT*[cdc25] (O'Connell *et al.,*

1992), *nimX*[cdc2] (Osmani *et al.,* 1994), *bimC3*[kinesin] (Enos and Morris, 1990), and *bimE*[Apc1] (James *et al.,* 1995). All double mutants grown for ≥24 h at 37°C arrested with single nuclei in poorly polarized cells (not shown). *hypA* may interact with *nimX*[cdc2] at Y305H, the site mutated in *nimX3: hypA1, nimX3* strains grew very slowly at 28°C, with wildtype hyphal morphology, and sporulation was delayed by about 2 days.

Double mutants were grown for 14 h at 28°C (24 h for *hypA1, nimX3*[cdc2]) and then kept at 28°C or shifted to 37 or 42°C for 1.5 or 3 h, before fixation. We used a 3-h upshift in case double mutants inserted septa more slowly at restrictive temperature than *hypA1* alone. T–S1 distances were measured as before. For the *hypA1, nim* double mutants, the T–S1 distance was similar between upshifted and control germlings (not shown). However, the T–S1 distance was shorter for upshifted *hypA1, bim* germlings than for controls kept at 28°C: *hypA1, bimC3,* 90 ± 5 μm (upshifted) vs 174 ± 5 μm (control); *hypA1, bimE7,* 99 ± 5 μm (upshifted) vs 161 ± 4 μm (control) (each, $n = 50 ± SE; P < 0.05,$ ANOVA).

Transmission electron microscopy showed that presumptive nuclear fragments adjacent to forming tip cell septa at 1.5 h after upshift had profiles and contents similar to those of wildtype interphase nuclei. Similarities included having electron-dense nucleoli, dispersed chromatin, and intact nuclear envelopes (not shown). However, these nuclei or fragments were unstable since they disappeared beginning about 2.5 h after upshift (Kaminskyj and Hamer, 1998; and data not shown). Neither cytochalasin A nor benomyl treatment prevented upshift-induced degradation of tip cell nuclei.

Plant cell division is analogous to that in fungi since cytokinesis is temporally and spatially correlated with mitosis, although plant cells divide by means of a phragmoplast rather than cell wall invagination. *hypA1* germlings were grown as before, transferred to medium amended with caffeine, which inhibits phragmoplast formation, and incubated for 1.5 h at 28 or 42°C. Unlike *Tradescantia* (Valster and Hepler, 1997), treating *hypA1* germlings with 2 mM caffeine did not inhibit tip cell septation (Fig. 4, $P <$ 0.05, ANOVA). Hyphal growth was severely inhibited at 28°C by ≥5 mM caffeine.

To test the dependence of tip cell septation on cell size, *hypA1* spores were grown at 28°C for 8.5 h and then shifted to 42°C for 1.5 h or kept at 28°C (Fig. 6, Table 2). At 8.5 h, *hypA1* germlings averaged 45 μm long and 8% had produced their first septum. After another 1.5 h, germlings had one to four septa whether they were shifted to 42°C or kept at 28°C. Germlings kept at 28°C grew

TABLE 2

Septation in Young *hypA1* Germlings

Growth regime	Germling length (μm) ± SD	Average number of nuclei ± SD	Number of septa (0:1:2:3:4)	Tip-first septum length (μm) ± SD[a]
8.5 h 28°C	44.6 ± 9.3	11.7 ± 3.7[b]	92:8:0:0:0	32.5 ± 6.4
(8.5 h 28°C + 1.5 h 28°C)	115.0 ± 35.8	—[c]	0:46:30:22:2	92.1 ± 28.2
(8.5 h 28°C + 1.5 h 42°C)	62.8 ± 21.1	—[d]	2:24:42:30:2	36.3 ± 17.3

[a] For germlings with septa.
[b] Range: 6–18.
[c] Many wildtype nuclei.
[d] Many nuclear fragments.

2.5-fold in length, whereas upshifted germlings grew relatively little and had fragmented nuclei (Fig. 6) that disintegrated later (not shown). Seven-hour-old *hypA1* germlings did not insert any septa after upshift.

Eventually, upshifted *hypA1* tip cells lost cell membrane integrity and died. Ethidium bromide has a high signal-to-background ratio when examined using epifluorescence optics. Nuclei and mitochondria in cells with damaged membranes stained brightly under these conditions with morphologies indistinguishable from those of fixed and Hoechst 33258-stained preparations. Ethidium bromide exclusion was unreliable once nuclear disappearance became prevalent, after which toluidine blue uptake was used. About 1% of control tip cells could be stained with ethidium bromide during the first 1.5 h after upshift, which likely represented damage during mounting. Between 2 and 3 h, this increased to about 5% of tip cells. By 3.5 h after upshift, half of the tip cells had lost cell membrane integrity.

DISCUSSION

In *A. nidulans,* a round of mitosis in multinucleate tip cells is followed by insertion of one of more septa (Fiddy and Trinci, 1976) at premarked sites (Trinci and Morris, 1979), creating basal cells about 40 μm long (Wolkow *et al.,* 1996; Kaminskyj and Hamer, 1998). Septa usually form between pairs of daughter nuclei (Momany and Hamer, 1997), and a single mitotic nucleus can trigger septation (Wolkow *et al.,* 1996). In mutants whose nuclear distribution is markedly uneven, septum distribution is uneven as well (Wolkow *et al.,* 1996), suggesting that mitotic signals have localized effects. However, these results do not explain why *A. nidulans* septa are evenly spaced, nor why basal cells contain three or four nuclei (Kaminskyj and Hamer, 1998).

When *A. nidulans* germlings containing the *hypA1* mutation are grown for at least 12 h at 28°C and then are shifted to 42°C, they insert septa into their tip cells with wildtype spacing. This is the first report of *A. nidulans* septation near the hyphal tip, suggesting that septa form at sites established early in hyphal growth, in advance of their normal activation in basal regions.

What evidence is there that tip cell septa are sufficiently like wildtype to be a credible model for spatial regulation? First, septation in *A. nidulans* follows mitosis (Harris *et al.,* 1994; Momany and Hamer, 1997), even in aseptate mutants shifted from restrictive to permissive temperature (Trinci and Morris, 1979; Harris *et al.,* 1994). Tip cell septation follows mitosis, albeit an aberrant one apparently triggered by the loss of *hypA1* product function. Benomyl blocks mitosis and septation in *A. nidulans* (Harris *et al.,* 1994; Momany and Hamer, 1997) and has a similar effect on tip cell septation. In a *hypA1* background, tip cell septation was blocked by *nim* mutations that arrest in interphase. Second, septation requires actin cytoskeleton function (Harris *et al.,* 1994; Momany and Hamer, 1997) and a minimum cell size (Harris *et al.,* 1994; Wolkow *et al.,* 1996), both of which are needed for tip cell septation. Third, both wildtype (Wolkow *et al.,* 1996; Kaminskyj and Hamer, 1998) and tip cell septa are evenly spaced, about 40 μm apart. Fourth, although tip cell septa appear to lack a central pore, like wildtype the tip cell septa are trilamellar and form by centripetal invagination. Fifth, tip cell septa are unlike retraction septa in *Basidiobolus* (Robinow, 1963) since *A. nidulans* hyphal tip cells have abundant cytoplasm in their subapical regions. Tip cell septa form in cells that retain their nuclei and cell membrane integrity for an hour or more after septum formation, and they are not induced by mechanical damage as is occasionally seen in oomycete hyphae (Kaminskyj, unpublished results). Finally, tip cell septation is not exclusive to *hypA1,* as it is also seen in upshifted *hypA6* and *hypB5* strains (Kaminskyj, unpublished results).
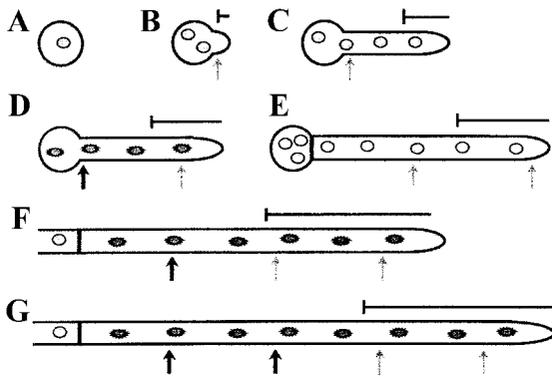
FIG. 7. Model for septum positioning in *Aspergillus nidulans*. An *A. nidulans* conidium (A) germinates (B), marks the first future site of septation (gray arrow), and produces a germ tube (C). Interphase nuclei are shown as light ovals. Mitotic nuclei (dark ovals in D, F, G) can activate (black arrow) nearby septation markers unless septation is inhibited (⊢) by proximity to the hyphal tip. In D, only the basal marker is activated, producing a septum at the spore to germ tube junction (E). Septation markers are established by an unknown mechanism as the hypha grows (light arrows in E–G), but only markers distal from the tip can be activated, which will result in insertion of one (F) or more (G) septa following mitosis.

Why was tip cell septation not observed when the *hypA1* upshift phenotype was first described (Kaminskyj and Hamer, 1998)? Tip cell septation is induced by upshift to 42°C, but not to even slightly higher temperatures. However, upshift to 37°C induced tip cell septation indistinguishable from a shift to 42°C (not shown). This is consistent with the results of Harris (1997), who suggested that septation is more temperature sensitive than other aspects of *A. nidulans* growth.

Wolkow *et al.* (1996) proposed that a cortical marker deposited at germination is used to identify the future site of the first septum and that a tip-high inhibitor gradient prevents activation of this site until the germling reaches a critical length. Assuming that marking the first and later septa uses a comparable mechanism, the inhibitor proposed by Wolkow *et al.* (1996) could prevent activation of subsequent markers in near-apical hyphal regions. The even, wildtype spacing of tip cell septa in upshifted *hypA1* strains suggests that there are septation site markers nearer to the hyphal apex than are activated during wildtype growth. To accommodate these data, the model of Wolkow *et al.* (1996) can be modified as shown in Fig. 7.

The future site of the first septum is marked at the spore/germ tube junction during germination (Figs. 7A and 7B; Wolkow *et al.,* 1996). As the germling grows it periodically marks future septation sites (Figs. 7D–7G).

Septum formation requires a minimum cell size so predivisional mitoses (Figs. 7B and 7C; Harris *et al.,* 1994; Harris and Hamer, 1995) do not trigger septation due to a tip-high inhibitor gradient (Wolkow *et al.,* 1996). Once the size threshold is reached, often during the four-nucleus stage, the first septation site is activated at the next round of mitosis (Fig. 7D; Wolkow *et al.,* 1996). Additional septation sites would be inhibited by proximity to the hyphal tip (Figs. 7D and 7E). Depending on the relative lengths of the tip cell and inhibitor gradient, subsequent rounds of mitosis could activate markers for one (Fig. 7F) or more (Fig. 7G) septa.

This model suggests that *A. nidulans* tip cells measure time and/or distance to generate periodic septation marks. The growth rate of wildtype germlings increases dramatically during the first 12 h after germination (Kaminskyj and Hamer, 1998). Consistent with this, septa near the spore in an *A. nidulans* germling are more closely spaced than later ones (Fig. 1E; and Kaminskyj, unpublished results). Thus, there seems to be a temporal component to septation site marking, which might be tied to the cell cycle.

Are there cases where a mitotic nucleus is not aligned with a septation marker, but still close enough to activate it? Momany and Hamer (1997) examined the spatial relationship of mitotic nuclei and septum development and found that early in septation, 19% of the actin rings that precede chitin deposition formed beside rather than between a pair of mitotic nuclei.

In summary, upshifted *hypA1* germlings insert septa into their tip cells, apparently triggered by an aberrant mitosis and suggesting that *hypA* may be part of a mitotic checkpoint. Tip cell septa have a wildtype spacing that suggests that future septation sites are formed during hyphal extension but are inhibited by proximity to the tip.

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