Copyright © 1999 by the Genetics Society of America

Aspergillus nidulans swo Mutants Show Defects in Polarity Establishment, Polarity Maintenance and Hyphal Morphogenesis

Michelle Momany, Patrick J. Westfall and Gretel Abramowsky

Department of Botany, University of Georgia, Athens, Georgia 30602

Manuscript received June 12, 1998
Accepted for publication October 15, 1998

ABSTRACT

When the spores of filamentous fungi break dormancy, they grow isotropically, adding cell wall material uniformly in every direction. Later they switch to polarized growth, with new material added to the tip of an emerging germ tube. To identify genes involved in the synthesis and localization of cell wall material in filamentous fungi, we screened a collection of temperature-sensitive *Aspergillus nidulans* mutants for swollen cells. We have isolated mutants representing eight genes involved in polarity establishment, polarity maintenance, and hyphal morphogenesis. On the basis of the results of temperature-shift experiments, *swo C*, *D*, and *F* are required to establish polarity, while *swoA* is required to maintain polarity. *swo B*, *E*, *G*, and *H* are involved in later hyphal morphogenesis. Our results suggest that polarity establishment and polarity maintenance are genetically separate events and that a persistent signal is required for apical extension in *A. nidulans.*

ALL cells must organize areas of new growth to maintain their shape and function properly. Fungi employ distinctive patterns of new cell wall addition that ultimately lead to their growth as unicells, in the case of yeasts, or as hyphae, in the case of filamentous fungi. These distinctive patterns of cell wall deposition are the visible result of changes in cellular polarity. Many details of the polarity establishment that determines localization of new cell wall material are known for the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* In contrast, the processes of polarity establishment and subsequent new cell wall synthesis and localization are relatively unexplored in filamentous fungi.

The budding yeast *S. cerevisiae* shifts between isotropic and polarized growth modes (Lew and Reed 1995; Roemer *et al.* 1996). The nascent bud emerges from a specific site on the cell surface and grows by apical extension, adding new material at the tip of the bud. After DNA replication is completed, this polar growth is replaced by isotropic growth and the bud begins to expand uniformly. Eventually, a septum separates the mother cell and daughter bud. Mother and daughter each begin the process again with emergence of a new bud in a polarized manner. Throughout this process, actin is concentrated in areas of growth where it is thought to allow transport of vesicles carrying materials needed to build the new cell wall. Several gene products required for the selection and organization of the new growth sites have been identified. These include actin, the septins, and products of the *BUD* genes.

Corresponding author: Michelle Momany, Department of Botany, 2502 Plant Sciences, University of Georgia, Athens, GA 30602. E-mail: momany@dogwood.botany.uga.edu

The fission yeast *S. pombe* also switches between two distinct growth patterns (Mitchison and Nurse 1985). Immediately after dividing by fission, new cells show unipolar growth, extending only from one end of the cell. Early in G_2, the cell switches to bipolar growth, extending from both ends. After cytokinesis, mother and daughter begin the cycle again with unipolar growth at one end. As is true for *S. cerevisiae*, actin is concentrated in the regions of new cell wall addition (Marks and Hyams 1985). Recently, two genes important for proper morphogenesis in *S. pombe* have been identified. The *tea1* and *pom1* gene products are found at the ends of the fission yeast at various times in the cell cycle and are thought to play a role in localization of the growth machinery (Mata and Nurse 1997; Bahler and Pringle 1998).

Like yeast, filamentous fungi employ two distinct growth modes. When spores of filamentous fungi break dormancy, they first grow isotropically, adding new cell wall material uniformly in every direction. Later they switch to polarized growth, with new cell wall material forming an emerging germ tube (Bartnicki-Garcia and Lippman 1969; reviewed by Harris 1997). This polarized growth continues with the addition of cell wall material exclusively at the tip of the germ tube, a process known as apical extension. Apical extension ultimately shapes cells into the elongated filaments, or hyphae, typical of many fungi. As is true for budding and fission yeast, actin is concentrated in the areas of new cell wall growth (reviewed by Heath 1990). The Spitzenkörper, a distinctive collection of vesicles, is also seen in the growing tip where it is thought to function as a "vesicle supply center," organizing the materials needed to build the new cell wall (Reynaga-Pena *et al.* 1997 and refer-

TABLE 1

***A. nidulans* strains**

Strain	Genotype
A28[a]	*pabaA6; biA1*
AH12[b]	*argB2; chaA-1*
AGA20	*swoD*
AGA24	*swoC*
AJB11	*swoF*
AJB15	*swoB*
AJB16	*swoA-2*
AJB17	*swoE*
APW14	*swoH*
APW18	*swoA-1*
APW22	*swoG*
ts1-67[c]	*swoB; pabaA6; biA1*
ts1-168[c]	*swoG; pabaA6; biA1*
ts6-90[c]	*swoA-1; pabaA6; biA1*
ts7-20[c]	*swoH; pabaA6; biA1*
ts7-28[c]	*swoD; pabaA6; biA1*
ts7-91[c]	*swoA-2; pabaA6; biA1*
ts71-99[c]	*swoF; pabaA6; biA1*
ts8-160[c,d]	*swoE; pabaA6; biA1*
ts10-4[c]	*swoC; pabaA6; biA1*

[a]Available from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center (Kansas City, KS).

[b]Obtained from John E. Hamer, Department of Biological Sciences, Purdue University (West Lafayette, IN).

[c]Original isolate from temperature-sensitive collection (Harris *et al.* 1994); Collection obtained from John E. Hamer, Department of Biological Sciences, Purdue University (West Lafayette, IN).

[d]Isolated independently in screens for hypercellular mutants (*hypA;* Kaminskyj and Hamer 1998) and polarity defective mutants (*podA;* S. D. Harris, personal communication).

ences therein). As pointed out by Harris *et al.* (1997), the critical difference in polarity establishment between yeasts and filamentous fungi is that the axis of polarity in yeasts reorients, but, once established, the axis of polarity in filamentous fungi remains fixed.

Although the signal(s) responsible for polarity establishment in filamentous fungi is still unknown, several molecules implicated in subsequent hyphal extension and morphogenesis have recently been identified. In *Neurospora crassa*, deletion of the microtubule-dependent motor protein kinesin results in cells that are shorter and fatter than wild type (Seiler *et al.* 1997) and a mutant with defects in the regulatory subunit of cAMP-dependent protein kinase shows hyphal swelling (Bruno *et al.* 1996). In *A. nidulans*, loss of type I myosin function leads to enlarged cells that cannot grow by apical extension (McGoldrick *et al.* 1995), and mutation of *sepA*, which encodes an FH 1/2 protein, results in cells with abnormally wide hyphae and no septa (Harris *et al.* 1997). Normal patterns of hyphal development can also be disrupted by mutations in *samB*, which encodes a novel Zn-finger-containing protein (Kruger and Fischer 1998).

TABLE 2

***swo* mutant characteristics**

Allele designation	Ts+:Ts−[a]	Dominant/ recessive[b]	Osmotic remedial[c]	Calcofluor hypersensitive[d]
swoA-1	50:50	R	42°	Yes
swoA-2	50:50	R	42°	Yes
swoB	50:50	R	42°	Yes
swoC	49:50	R	37°	No
swoD	53:47	R	37°	No
swoE[e]	44:52	R	37°	No
swoF	53:47	R	42°	No
swoG	47:53	R	42°	No
swoH	45:55	R	No	No

[a]Temperature sensitivity of progeny from mutant by AH12 cross.

[b]D, dominant; R, recessive; based on phenotype of *swo*/wild-type diploid.

[c]Based on ability of 1 m sucrose to restore wild-type growth on solid medium at semipermissive (37°) or restrictive temperature (42°) relative to growth at the same temperature without sucrose; see Figure 1.

[d]Based on ability of 0.1 mg/ml Calcofluor to inhibit growth relative to wild type at permissive temperature (30°). None of the *swo* mutants was resistant to Calcofluor at 0.25 mg/ml (data not shown); see Figure 1.

[e]Isolated independently in screens for hypercellular mutants (*hypA;* Kaminskyj and Hamer 1998) and polarity defective mutants (*podA;* S. D. Harris, personal communication).

To identify genes involved in the synthesis and localization of new cell wall material in filamentous fungi, we screened a collection of temperature-sensitive *A. nidulans* mutants for swollen cells. Defects in either cell wall localization or synthesis can presumably disrupt morphogenesis, giving rise to misshapen, swollen hyphae. Because cell wall localization ultimately depends upon cell polarity, we expected that at least some of our mutants would have polarity defects. We have isolated mutants representing eight genes involved in polarity

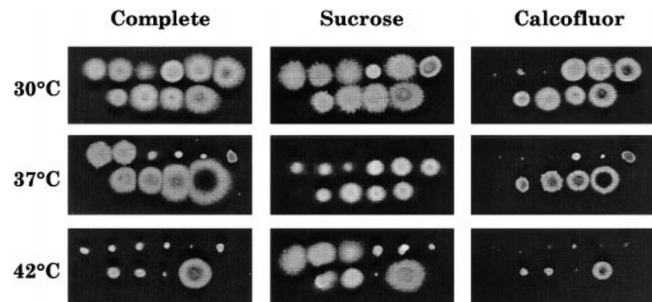

Figure 1.—Phenotypes of *swo* mutants on solid medium. The *swo* mutants were inoculated to complete medium, complete medium with 1 m sucrose, or complete medium with 0.1 mg/ml Calcofluor, and incubated for 2 days at permissive temperature (30°), semipermissive temperature (37°), or restrictive temperature (42°), as indicated. Top row (from left to right): APW18 (*swoA-1*), AJB16 (*swoA-2*), AJB15 (*swoB*), AGA24 (*swoC*), AGA20 (*swoD*), AJB17 (*swoE*); second row (from left to right): AJB11 (*swoF*), APW22 (*swoG*), APW14 (*swoH*), A28 (wild type).

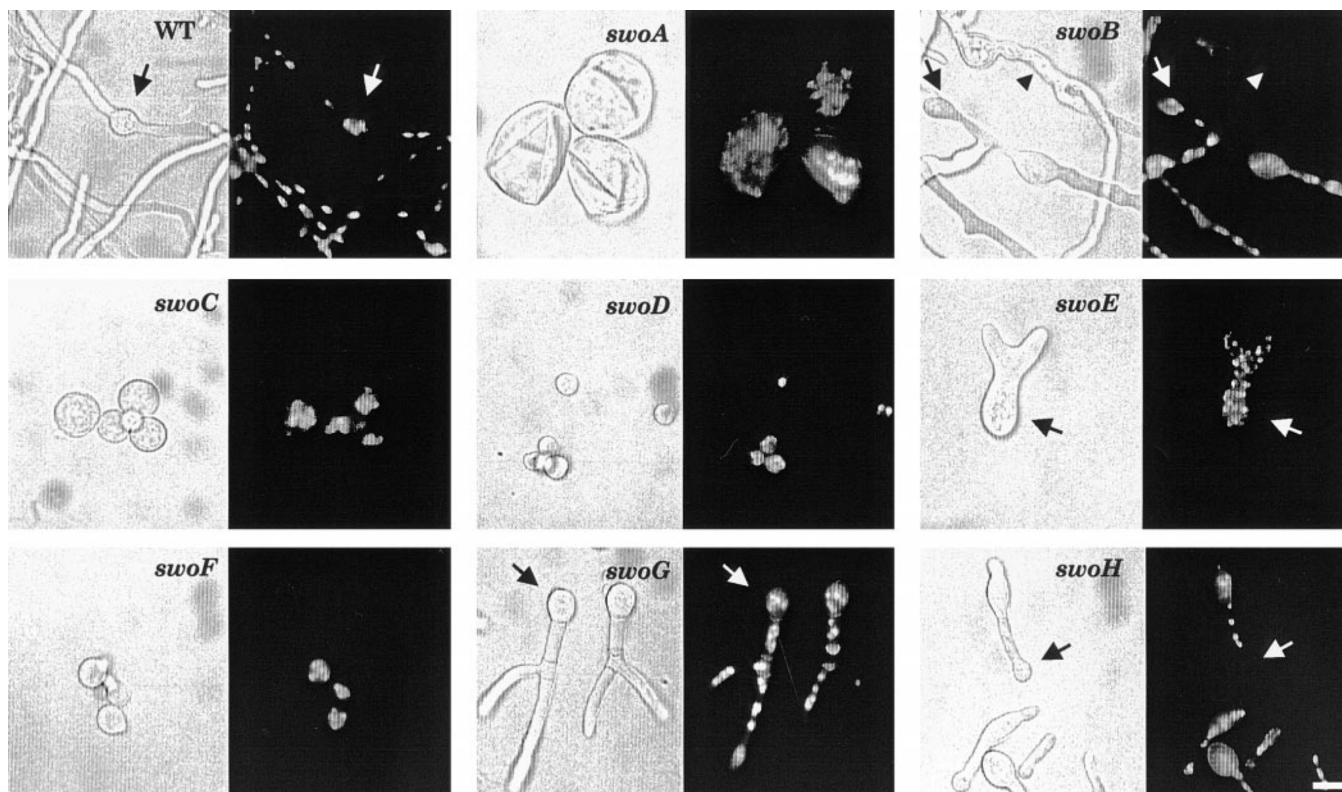

Figure 2.—Phenotypes of *swo* mutants in liquid medium. The *swo* mutants were inoculated onto coverslips in complete liquid medium and incubated at restrictive temperature for 13 hr. Germlings were fixed and stained with Hoescht 33258 to visualize nuclei. The left and right panels show phase contrast and fluorescent images of the same field. Arrows mark conidia from which germ tubes emerged. Arrowhead marks an empty hypha, frequently seen in *swoB* strains. All strains are as listed for Figure 1 except that *swoA-1* is not shown. All micrographs are at the same magnification. Bar, 10 μm.

establishment, polarity maintenance, and hyphal morphogenesis. Our results suggest that polarity establishment and polarity maintenance are separate events and that a persistent signal is required for apical extension.

MATERIALS AND METHODS

Aspergillus strains and growth methods: Strains used in this study are listed in Table 1. Generation of the temperature-sensitive mutant collection has been previously described (Harris *et al.* 1994). Media used were complete medium (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5), or minimal medium (1% glucose, nitrate salts, trace elements, pH 6.5). Trace elements, vitamins, nitrate salts, and amino acid supplements are described in the appendix to Kafer (1977). For solid media 1.8% agar was added.

Strain construction and genetic analysis were by standard *A. nidulans* techniques (Kafer 1977; Harris *et al.* 1994). In addition to strains listed in Table 1, *swo* mutants with *pabaA6* or *argB2* markers were generated by crossing the original temperature-sensitive *swo* isolates listed in Table 1: (*swo*; *pabaA6; biA1*) to AH12 (*argB2; chaA-1*). *Swo* strains with reciprocal *pabaA6* and *argB2* markers were used for diploid construction and crosses.

Staining and microscopy: Examination of phenotypes in liquid medium has been previously described by Harris *et al.* (1994). Complete liquid medium (10 ml) was inoculated with 1–5 × 10^4 conidia/ml, poured into a Petri dish containing a glass coverslip, and incubated at 42° for 12–16 hr. Coverslips with adhering germlings were fixed in 3.7% formaldehyde, 50 mm phosphate buffer (pH 7.0), and 0.2% triton for 30–60 min. Coverslips were then washed with water, incubated 5 min with 10 μg/ml Calcofluor (American Cyanamid, Wayne, NJ) and 100 ng/ml Hoechst 33258 (Sigma, St. Louis), washed again, and mounted on a microscope slide for viewing. Germlings were photographed using a Zeiss (Thornwood, NY) Axioplan microscope and Zeiss MC100 microscope camera system with Kodak (Rochester, NY) Tmax 100 film.

Localization of cell wall addition: We initially attempted to label cell walls with the lectin wheat germ agglutinin coupled to fluorescein isothiocyanate (FITC; Sigma). In multiple experiments, the lectin did not label a band at the basal end of the germ tube. Therefore, we raised antibodies against total cell walls for use in labeling experiments. *A. nidulans* A28 was grown for 48 hr in complete liquid medium. Hyphae were homogenized and walls were isolated as previously described (Bull 1970). Antibodies were produced by the Monoclonal Antibody Production Facility at the University of Georgia. Mice were inoculated with 50 μg of cell walls in incomplete Freund adjuvant and boosted four times as previously described (Ste-Marie *et al.* 1990). Serum was collected and shown to contain antibodies against *A. nidulans* cell walls by an ELISA assay using cell walls as antigen.

For data presented in Figure 3, conidia were inoculated onto coverslips in 10 ml of complete liquid medium and incubated at 30° for 4, 5, 6, or 7 hr. Coverslips with adhering germlings were removed from liquid medium and incubated with anticell wall antibody (diluted 1:1000 in phosphate buffered saline) at room temperature for 30 min. Coverslips with adhering hyphae were then washed three times in phosphate buffered saline, transferred to fresh complete liquid medium,

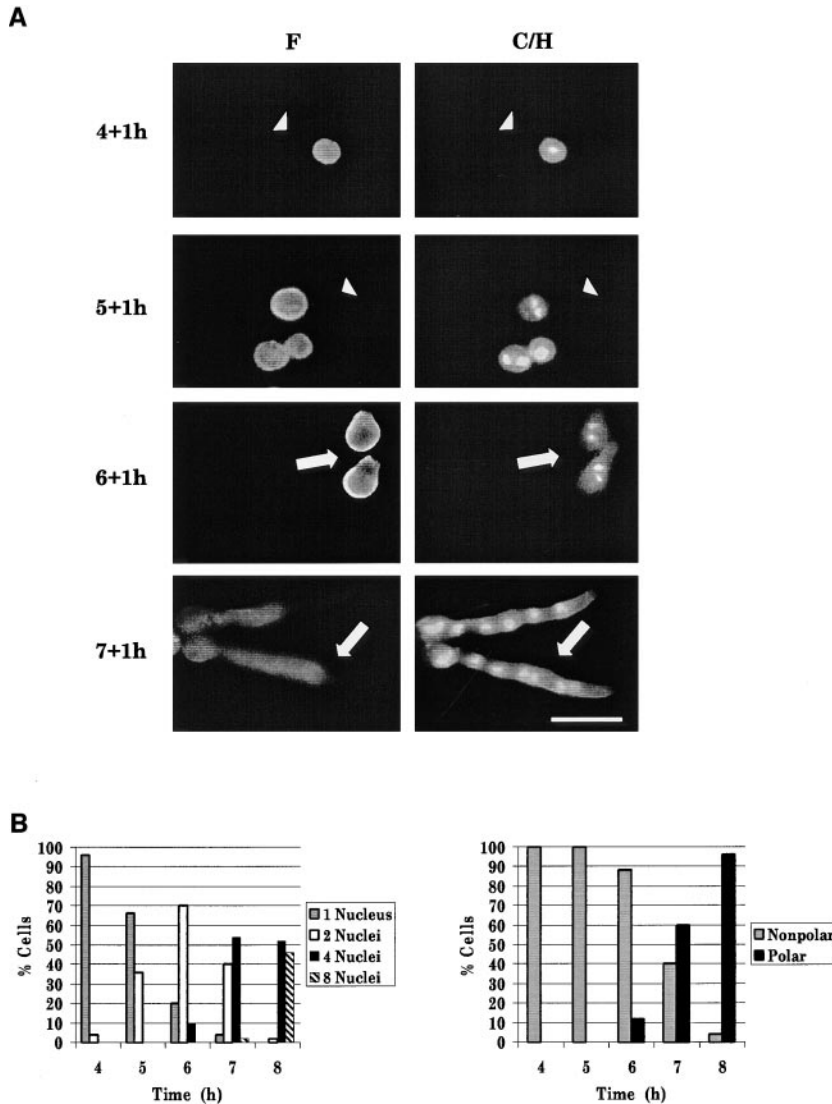

Figure 3.—The switch from isotropic to polar growth. Conidia of wild-type *A. nidulans* (A28) were inoculated onto coverslips in complete medium, grown for 4, 5, 6, or 7 hr, incubated with polyclonal antibodies against cell walls, washed, transferred to fresh medium without antibodies, and incubated for another hour. They were then fixed and stained with an FITC-labeled secondary antibody, Calcofluor to visualize chitin, and Hoescht 33258 to visualize nuclei. (A) Left column shows FITC staining (labels growth that occurred prior to the last hour of incubation). Right column shows Calcofluor and Hoescht 33258 staining (labels entire hypha and nuclei). Arrowheads denote examples of cells that grew in a nonpolar manner during the final hour of incubation. Arrows denote examples of germlings that grew in a polar manner during the final hour of incubation. All micrographs are at the same magnification. Bar, 10 μm. (B) Nuclear number and growth polarity were scored for 50 germlings for each time point. Cells that showed uniform antibody staining were scored as nonpolar. Cells that showed a localized unlabeled zone were scored as polar. Experiment was repeated three times with essentially identical results. A typical data set is shown.

and incubated for an additional hour at 30°. Coverslips with adhering germlings were then fixed, stained with FITC-coupled anti-mouse mouse antibody (Sigma), washed three times in phosphate buffered saline, stained with Calcofluor and Hoescht 33258, and placed on a microscope slide for viewing. In control experiments omitting the antibodies against cell walls (primary antibody), no staining was seen. In controls omitting the final hour of incubation, the entire germling was labeled. In controls delaying treatment with antibodies against cell walls until after the final hour of incubation, the entire germling was labeled. For data presented in Figure 3, 50 germlings were counted for each time point. Experiments were repeated three times with essentially identical results. A typical data set is shown.

Temperature-shift experiments: Temperature-shift experiments were carried out as described above (staining and microscopy) except for changes in incubation times and temperatures. For downshift experiments (Figure 4), conidia were inoculated to complete medium at 42°, incubated for 10 hr, and transferred to 30° for 2 hr prior to fixing and staining. For upshift experiments (Figure 5) conidia were inoculated to complete medium at 30°, incubated for 10 hr, and transferred to 42° for 2 hr prior to fixing and staining. Experiments were repeated three times with essentially identical results.

Upshift experiments with localization of new growth (Figure 6) were carried out as described above with the following modifications. Conidia were incubated for 11.5 hr at 30°, treated with antibodies against cell walls for 30 min, transferred to fresh media prewarmed to 42°, and incubated for 4.5 hr at 42° prior to processing as described for localization of cell wall addition. When wild type was treated identically, growth was more rapid, making photography of an entire germling at high magnification difficult. For the wild-type control shown, incubation times were shortened to 10 hr at permissive temperature and 2 hr at restrictive temperature.

RESULTS

Identification of *swo* mutants: To identify genes important in cell wall synthesis and localization, we screened a preexisting collection of 1200 temperature-sensitive *A. nidulans* mutants (Harris *et al.* 1994) for strains that showed a swollen cell phenotype on solid medium at restrictive temperature. Fifty-five strains were identified as putative swollen cell (*swo*) mutants on the basis of an initial stereoscopic examination. The puta-

TABLE 3

Nuclear number for nonpolar *swo* mutants

Mutant	Average nuclear number[a]	High nuclear number	Low nuclear number	Nuclear morphology[b]
swoA-2	40	108	13	Normal
swoC	5.8	12	2	Elongated, stringy
swoD	5.2	10	2	Normal
swoF	5.3	12	2	Elongated, stringy

[a] Conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 13 hr, fixed, and stained with Hoescht's 33258 to visualize nuclei. Nuclei in 50 germlings were counted. Two independent experiments gave similar results. The average, high, and low nuclear numbers represent all 100 germlings counted. Because *swoA* and *swoC* mutants are thick, accurate nuclear counts are difficult (see Figure 2). Therefore, counts for these strains may underrepresent the total nuclear number.

[b] Abnormal nuclear morphologies in *swoC* and *swoF* made accurate nuclear counts difficult. Therefore, counts for these strains may underrepresent the total nuclear number.

tive *swo* mutant phenotypes ranged from large, round cells with small germ tubes to hyphae with distended, bulging regions (data not shown).

Genetic characterization: Because *A. nidulans* is haploid, single gene mutations are expected to segregate 1:1 in crosses with wild type. The 55 putative *swo* mutants were crossed to wild type and progeny were scored for temperature sensitivity and swelling. Nine mutants showed both 1:1 segregation and linkage of temperature sensitivity to the swollen cell phenotype (Table 2).

To determine whether the *swo* mutations are dominant or recessive, stable diploids were constructed with wild-type strains. All diploids were wild type at restrictive temperature, indicating that the *swo* mutations are recessive (Table 2).

To determine if any of the nine *swo* mutations are allelic or tightly linked, crosses were made for every pairwise mutant combination using *swo* mutant strains containing reciprocal auxotrophic markers. The recovery of wild-type progeny from a cross was interpreted to mean that each parent strain had a lesion in a different *swo* gene. Wild-type progeny were recovered from every cross except those derived from ts6-90 by ts7-91 (data not shown). Stable diploid strains were constructed from fusion of ts6-90 and ts7-91 derivatives to determine whether they represented alleles or tightly linked genes. All ts6-90/ts7-91 diploids were temperature sensitive, indicating that the respective haploids are allelic. Ts6-90 and ts7-91 were designated *swoA-1* and *swoA-2*, respectively. The seven other *swo* mutants were designated *swoB*–*swoH* (Tables 1 and 2).

Phenotypic characterization: The phenotypes of the *swo* mutants on solid medium were observed by inoculating to complete solid medium and incubating for 2 days at restrictive temperature. All mutants were able to send out germ tubes, although both alleles of *swoA* made only a few. Most of the *swo* mutants did not make the asexual spores (conidia) typically seen in wild-type strains after 2 days growth, although sparse conidiation was visible in cultures of *swoB*, *swoD*, and *swoF.* Many morphological mutants show wild-type growth when extra osmoticum is added to the medium. This restoration to the wild-type phenotype, known as osmotic remediality, is especially likely in the case of mutants where a weakened wall cannot resist internal turgor pressure. The *swo* mutants were tested for osmotic remediality on solid medium containing high levels of sucrose, sorbitol, or potassium chloride. Sucrose was the best osmotic stabilizer and so only those results are reported (Figure 1, Table 2, and data not shown). Five of the mutants (*swoA-1*, *A-2*, *B*, *F*, and *G*) were osmotically remedial at the restrictive temperature. Three (*swoC*, *D*, and *E*) were osmotically remedial at the semipermissive temperature. Only one mutant showed no osmotic remediality (*swoH*).

The dye Calcofluor binds to β-linked glucans and chitin (Maeda and Ishida 1967). Some classes of cell wall mutants are resistant to the dye (Elorza *et al.* 1983; Roncero *et al.* 1988). By growing *A. nidulans* on media containing various concentrations of Calcofluor, we determined that wild type is resistant to Calcofluor at 0.1 mg/ml, but is sensitive to Calcofluor at 0.25 mg/ml (data not shown). Three of the *swo* mutants could not grow well in the presence of 0.1 mg/ml Calcofluor and thus are Calcofluor hypersensitive (*swoA-1*, *A-2*, and *B*; Figure 1, Table 2). None of the mutants were able to grow in the presence of 0.25 mg/ml Calcofluor (data not shown). Thus, none of the *swo* mutants are Calcofluor resistant.

The phenotypes of the *swo* mutants in liquid were observed by inoculating on coverslips in complete medium and incubating at permissive or restrictive temperature (Figure 2). After 12–16 hr incubation, germlings were fixed and stained with Calcofluor and Hoescht 33258 to stain chitin and nuclei, respectively. At permissive temperature all *swo* mutants were indistinguishable from wild type, except for *swoB.* Only 26% of *swoB* germlings had septa after overnight growth at permissive temperature, compared to 100% in wild-type control cultures. At restrictive temperature, four of the *swo* mutants did not generally form germ tubes, instead showing isotropic or nonpolarized growth (*swoA*, *C*, *D*, and *F*; Figure 2, Table 4). The *swoA-1* allele occasionally formed germ tubes, while *swoA-2* never did. We postulate that *swoA-1* is a leaky allele. The other mutants grew in a polar manner, but exhibited hyphal swelling or other morphological abnormalities (*swoB*, *E*, *G*, and *H*; Figure 2, Table 4).

The switch from isotropic to polar growth in wild type: When wild-type *A. nidulans* conidia germinate, they first expand uniformly in every direction and later

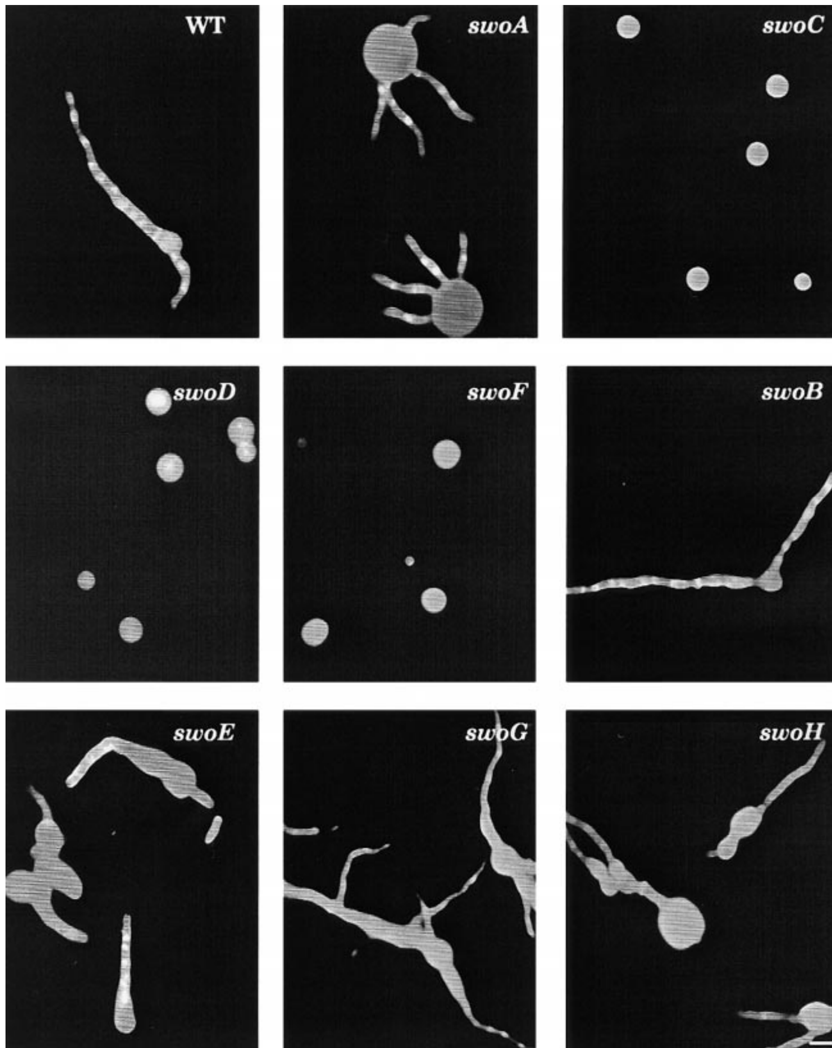

Figure 4.—Phenotypes of *swo* mutants shifted from restrictive to permissive temperature. Conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hr, shifted to permissive temperature, incubated for another 2 hr, fixed, and stained with Calcofluor and Hoescht 33258. Much of the growth of *swo* mutants at restrictive temperature is so Calcofluor-bright that nuclei are not visible. All strains are as listed for Figure 1 except that *swoA-1* is not shown. All micrographs are at the same magnification. Bar, 10 μm.

grow by addition of new cell wall material at the tip of the germ tube. To define the timing of this switch from isotropic to polar growth relative to nuclear divisions, conidia of wild-type *A. nidulans* were inoculated onto coverslips in complete medium, grown for 4, 5, 6, or 7 hr, and incubated with polyclonal antibodies against cell walls for 30 min. Germlings were then washed, transferred to fresh medium without antibodies, and incubated for another hour. They were then fixed and stained with a fluorescently labeled secondary antibody (to label all growth prior to the final hour of incubation), Calcofluor (to label chitin), and Hoescht 33258 (to label nuclei). Cells that showed uniform antibody staining were scored as growing in a nonpolarized manner during the final hour of incubation. Cells that showed a localized unlabeled zone were scored as growing in a polarized manner during the final hour of incubation. Nuclear number and growth polarity were scored for each time point. As shown in Figure 3, the switch from isotropic to polar growth occurred at about 7 hr, around the time of the second nuclear division. Most cells with two nuclei grew isotropically, although occasionally a cell with only two nuclei grew in a polar manner. All cells with four nuclei grew in a polar manner. During the eighth hour of incubation, when most cells had eight nuclei, the germ tube grew by apical extension and the first septa were seen (data not shown). All of the *swo* mutants have at least four nuclei at restrictive temperature (Figure 2, Table 3) and so should be able to grow in a polar manner.

Temperature-shift experiments: To determine the effect of the *swo* mutations on polarity establishment, conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hr, shifted to permissive temperature, and incubated for another 2 hr (Figure 4, Table 4). We thought that the defect in polarity establishment would also prevent germ tube extension and so expected that the mutants that do not show polarized growth at restrictive temperature (*swoA*, *C*, *D*, and *F*) would not be able to make germ tubes upon release from the temperature block. Indeed, *swoC*, *D*, and *F* behaved as expected: they did not make germ tubes within 2 hr of the shift to permissive temperature. The *swoA* mutant, however, did make germ tubes after

TABLE 4

Polarization of *swo* mutants

Mutant	G.T.[a] at 42°?	Downshift[b] (42° → 30°)	Upshift[c, d] (30° → 42°)
swoA-1	No	Multiple hyphae, "axial"[e]	Basal[f] swells, new apical[g] growth swells
swoA-2	No	Multiple hyphae, "axial"	Basal swells, no new apical growth
swoC	No	No hyphae	Basal swells slightly, new apical growth normal
swoD	No	No hyphae	Basal swells slightly, new apical growth normal
swoF	No	No hyphae	Basal swells slightly, new apical growth swells
swoB	Yes	Basal swells slightly, apical normal	Basal swells, apical swells slightly, abnormal branching
swoE	Yes	Basal swells, apical normal	Basal swells, apical swells at tip
swoG	Yes	Basal swells, apical normal, hyperbranching	Basal normal, apical normal
swoH	Yes	Basal swells, apical normal	Basal normal, apical normal

[a] Germ tube; see Figure 2.
[b] Conidia were inoculated onto coverslips in liquid medium, incubated at restrictive temperature for 10 hr, shifted to permissive temperature, and incubated for another 2 hr; see Figure 4.
[c] Conidia were inoculated onto coverslips in liquid medium, incubated at permissive temperature for 10 hr, shifted to restrictive temperature, and incubated for another 2 hr; see Figure 5.
[d] For the *swo* mutants that do not make germ tubes at 42°, antibodies against total *A. nidulans* cell walls were used to distinguish growth that occurred before and after the temperature shift; see Figure 6.
[e] All germ tubes emerged from one side of the conidium. Whether these germ tubes arise sequentially and immediately adjacent to each other has not yet been determined.
[f] The older end of the germling, the conidium end.
[g] The newer end of the germling, the hyphal tip end.

the shift from restrictive to permissive temperature (Figure 4, Table 4). In fact, each cell made 3–4 normal looking germ tubes. Interestingly, these germ tubes were almost exclusively on one-half of the cell, rather than being equally spaced. Ninety-five percent of germ tubes formed in *swoA-1* and 100 percent of germ tubes formed in *swoA-2* after the downshift emerged from only one-half of the cell (Figure 4 and data not shown). As expected, the four *swo* mutants that showed polarized growth at restrictive temperature (*swoB*, *E*, *G*, and *H*) showed normal apical growth of hyphae after the shift from restrictive to permissive temperature (Figure 4, Table 4).

To determine the effect of the *swo* mutations on hyphal elongation, conidia were inoculated onto coverslips in complete medium, incubated at permissive temperature for 10 hr, shifted to restrictive temperature, and incubated for 2 hr. We expected that only the region of the hypha that grew after the shift to restrictive temperature (*i.e.*, the apical region) would swell. Surprisingly, none of the *swo* mutants showed exclusively apical swelling after the shift to restrictive temperature (Figure 5, Table 4). Both *swoA-1* and *A-2* showed pronounced swelling along the whole hypha. *SwoC* and *D* showed slight basal swelling and apparently normal apical growth. *SwoB* showed basal swelling with normal apical growth, while *swoE* showed basal swelling and apical swelling at the tip. *SwoG* and *H* looked normal in both basal and apical regions.

To better distinguish growth that occurred at permissive temperature from growth that occurred after the shift to restrictive temperature, conidia from the nonpolar *swo* mutants were inoculated onto coverslips in complete medium, grown at permissive temperature for 11.5 hr, and incubated with polyclonal antibody against *A. nidulans* cell walls for 30 min. The germlings were then washed and transferred to fresh medium without antibodies, incubated for 4.5 hr at restrictive temperature, fixed, and stained with fluorescently labeled secondary antibody, Calcofluor, and Hoescht 33258 (Figure 6). *SwoA-1*, *swoA-2*, and *swoF* clearly showed swelling in the region of the hypha that had grown at permissive temperature (basal). In addition, *swoA-1* (the presumed leaky allele) and *swoF* showed swollen growth in the region of the hypha that formed at restrictive temperature (apical). The *swoA-2* allele showed no new apical extension after the shift to restrictive temperature. *SwoC* and *D* both showed normal apical extension after the shift to restrictive temperature.

DISCUSSION

Phenotypes of the *swo* mutants: To identify genes involved in the synthesis and localization of new cell wall growth, we screened a collection of temperature-sensitive *A. nidulans* mutants for a swollen cell phenotype. Because the ultimate expression of polarity decisions is the localized deposition of new cell wall material, we expected that at least some of our *swo* mutants would have defects in polarity. We identified nine *swo* mutants

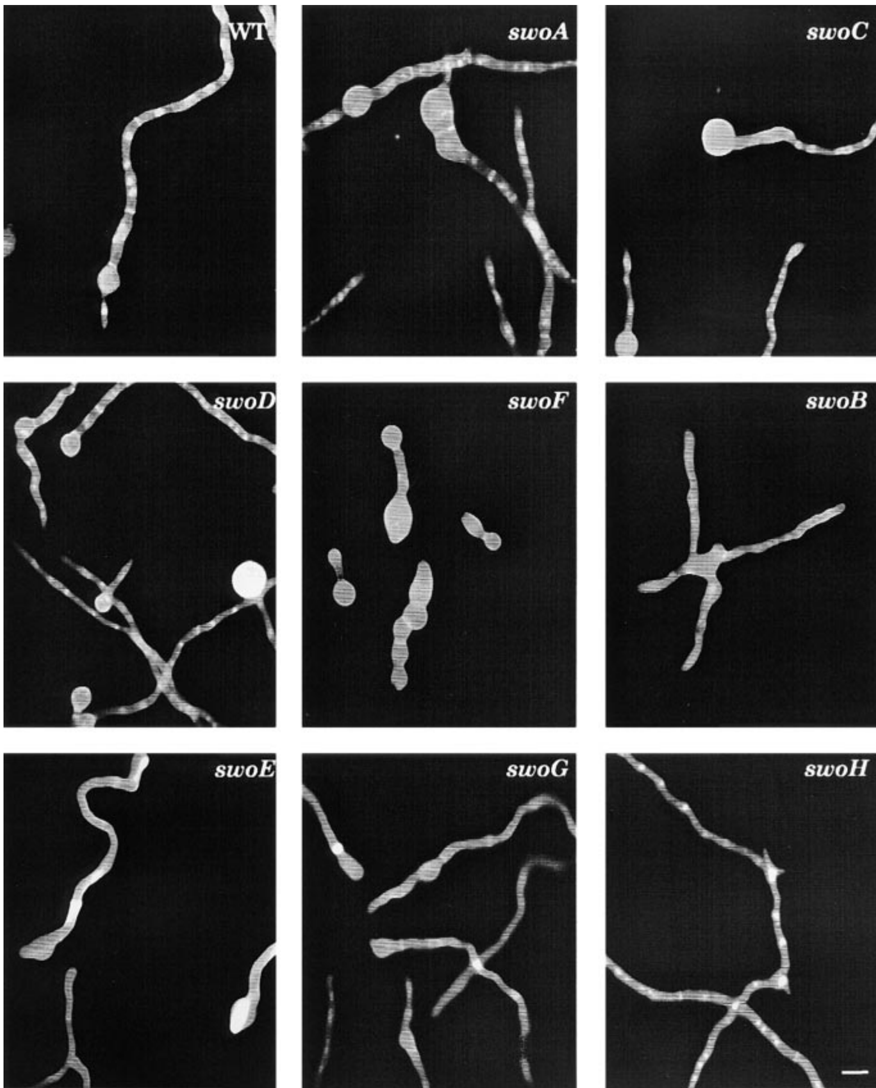

Figure 5.—Phenotypes of *swo* mutants shifted from permissive to restrictive temperature. Conidia were inoculated onto coverslips in liquid medium, incubated at permissive temperature for 10 hr, shifted to restrictive temperature, and incubated for another 2 hr, fixed, and stained with Calcofluor and Hoescht 33258. Much of the growth of *swo* mutants at restrictive temperature is so Calcofluor-bright that nuclei are not visible. All strains are as listed for Figure 1 except that *swoA-1* is not shown. All micrographs are at the same magnification. Bar, 10 μm.

representing eight genes. With the exception of *swoH*, the mutants show restoration of the wild-type phenotype by the addition of high osmoticum to the medium, hypersensitivity to the cell wall-binding dye Calcofluor, or both (Figure 1 and Table 2). These phenotypes are common among wall-related fungal mutants (Livingston 1969; Borgia and Dodge 1992; Borgia *et al.* 1996). The morphologies of the *swo* mutants fall into two broad categories: those that exhibit nonpolar growth in liquid medium at restrictive temperature (*swoA*, *C*, *D*, and *F*) and those that exhibit polar growth in liquid medium at restrictive temperature (*swoB*, *E*, *G*, and *H*; Figure 2, Table 4).

Nonpolar *swo* mutants show defects in polarity establishment and maintenance: It seemed likely that the nonpolar *swo* mutants were defective in the transition from isotropic to polar growth. In time course experiments we found that the isotropic-to-polar switch occurred near the second nuclear division (Figure 3). This is consistent with the previous observation that the germ tube emerges concomitant with the second nuclear division in *A. nidulans* (Harris *et al.* 1994). All of the *swo* mutants have at least four nuclei at restrictive temperature and therefore should be capable of growing in a polar manner (Figure 2, Table 3).

When incubated at restrictive temperature for 10 hr and then shifted to permissive temperature, *swoA* made germ tubes within 2 hr, while *swoC*, *D*, and *F* did not (Figure 4, Table 4). We interpret this to mean that *swoC*, *D*, and *F* are unable to establish polarity at restrictive temperature, while *swoA* is able to establish polarity but is blocked in the maintenance of polarity required for germ tube emergence. This result was surprising because there has been no previous evidence that polarity establishment can be separated from polarity maintenance in *A. nidulans.* We had assumed that the same signal would be responsible for both events, and so expected that none of the nonpolar *swo* mutants would be able to make germ tubes after release of the temperature block. An alternate interpretation is that both alleles of *swoA* are reversible. This seems unlikely because germ tubes can be seen emerging from *swoA* as early

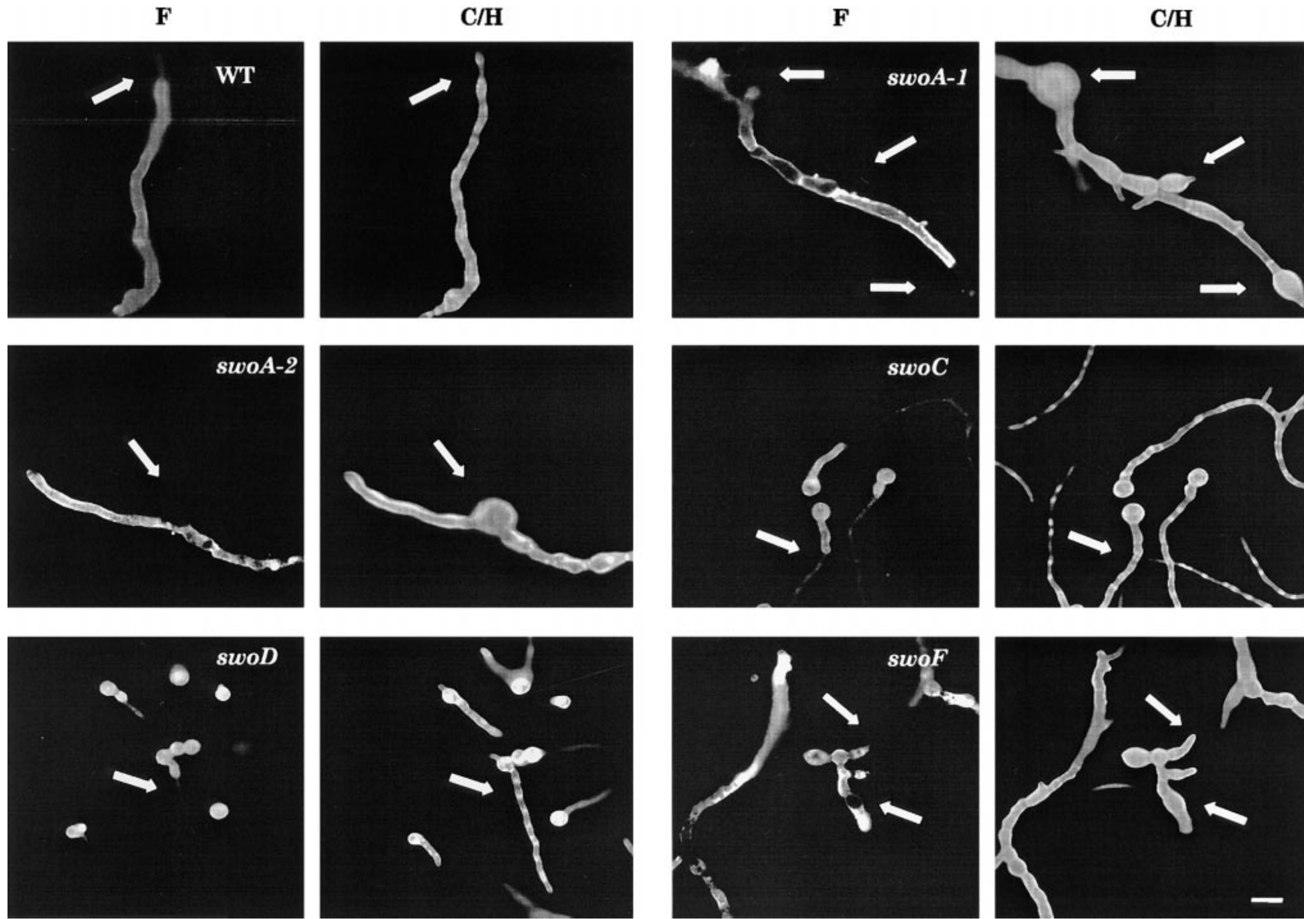

Figure 6.—Localization of growth in nonpolar *swo* mutants shifted from permissive to restrictive temperature. Conidia from the nonpolar *swo* mutants were inoculated onto coverslips in complete medium, grown at permissive temperature for 11.5 hr, incubated with polyclonal antibodies against cell walls, transferred to fresh medium without antibodies, and incubated for another 4.5 hr at restrictive temperature. Germlings were then fixed and stained with fluorescently labeled secondary antibody, Calcofluor, and Hoescht 33258. When a wild type was treated identically, growth was clearly by apical extension, but was much more rapid, making photography of an entire germling impossible at this magnification. For the wild-type control shown, incubation times were shortened to 10 hr at permissive temperature and 2 hr at restrictive temperature. Left column shows FITC staining (labels growth that occurred at permissive temperature). Right column shows Calcofluor and Hoescht 33258 staining (labels entire hypha and nuclei). Arrows mark examples of regions that grew after the switch to restrictive temperature. All strains are as listed for Figure 1. All micrographs are at the same magnification. Bar, 10 μm.

as 15 min after the shift from restrictive to permissive temperature (data not shown).

The pattern of germ tube emergence after release of the *swoA* temperature block was also a surprise. In wild-type *A. nidulans*, the first germ tube emerges and grows before a second germ tube emerges. This second germ tube is made 180° opposite the first (M. Momany, unpublished observations) in a pattern analogous to the bipolar budding pattern of diploid *S. cerevisiae* (reviewed by Roemer *et al.* 1996). The three to four germ tubes made by *swoA* after release of the temperature block all emerged from one-half of the cell (Figure 4). This unipolar pattern is reminiscent of axial budding in haploid *S. cerevisiae*, although whether the *swoA* germ tubes emerge sequentially or immediately adjacent to each other is not yet known. There are at least three possible explanations for the emergence of multiple germ tubes on one side of the cell. It could mean that the signal that makes the cell surface "competent" for germ tube emergence is overproduced or able to diffuse in *swoA*, and thus a larger area of the cell surface is competent for germ tube emergence. Or, perhaps a large portion of the cell surface is competent to send out germ tubes in wild type, but there is only enough of a germ tube inducer to cause a single germ tube to be made. In this scenario, it is the germ tube inducer that is overproduced in *swoA.* Alternatively, the *swoA* product may have a role in establishing the bipolar pattern or in suppressing the "axial" pattern in addition to its role in germ tube emergence.

When incubated at permissive temperature for 10 hr and shifted to restrictive temperature for 2 hr, *swoA*, *C*, *D*, and *F* showed varying levels of swelling in the basal region of the hypha (Figure 5, Table 4). Once more this

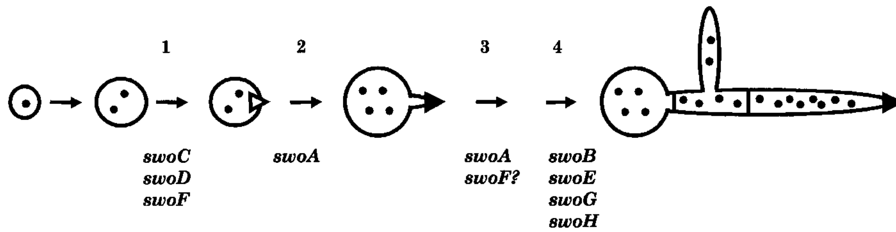

Figure 7.—Model of polarity establishment, polarity maintenance, and hyphal morphogenesis. Sometime between the first and second nuclear divisions the conidium switches from isotropic to polar growth. Presumably, a molecular marker tags the site from which the germ tube will emerge (open triangle). A separate signal, or perhaps a modification of the polarity establishment marker, is sent to initiate germ tube emergence and apical extension (closed triangle). As the hypha matures, it forms septa and branches. See text for postulated roles of the *swo* gene products.

result was a surprise. Most models of apical extension include the assumption that the cell wall in the basal region is set, that only the apical region is capable of growth. We expected that only the region of the hypha that formed at restrictive temperature (the apical region) would swell. Only *swoF* showed obvious swelling at the apex. Using antibodies against cell walls and longer incubation times, we were able to distinguish growth that occurred before and after the temperature shift more clearly (Figure 6). Our results showed that both alleles of *swoA* and *swoF* swelled in apical regions, with *swoA-2* also ceasing apical extension completely at restrictive temperature. Both *swoC* and *D* showed only slight basal swelling and made normal germ tubes after the shift to restrictive temperature. We interpret these results to mean that a persistent signal is required for polarity maintenance in *A. nidulans* and that the *swoA-2* mutant lacks this signal at restrictive temperature. The *swoA-1* and *swoF* mutants appear to be partially defective in polarity maintenance, because they are capable of some apical extension at restrictive temperature. The *swoC* and *D* mutants, on the other hand, appear to have no defect in apical extension once polarity has been established.

Polar *swo* mutants show defects in hyphal morphogenesis: The remaining *swo* mutants (*swoB*, *E*, *G*, and *H*) appear to be able to establish and maintain polarity at restrictive temperature despite defects in hyphal morphogenesis (Figure 2). The *swoB* mutant swells irregularly along the hypha and often ruptures, leaking cellular contents. This phenotype might indicate a defect in cell wall synthesis or assembly. The idea that *swoB* has a wall synthesis defect is consistent with its osmotic remediality and Calcofluor hypersensitivity (Figure 1, Table 2). The phenotype of the *swoE* mutant suggests a defect in branching and/or general hyphal patterning. Indeed, *swoE* has been isolated independently in screens for mutants with defects in cell patterning (*hypA*; Kaminskyj and Hamer 1998) and in polarity (*podA*; S. D. Harris, personal communication). The *swoG* mutant shows dichotomous branching at restrictive temperature, once more suggesting a defect in hyphal patterning. The *swoH* mutant shows frequent Calcofluor-bright areas along the hypha, possibly indicating overproduction or mislocalization of wall material disrupting normal hyphal patterning.

When incubated at restrictive temperature for 10 hr and then shifted to permissive temperature, all of the polar *swo* mutants showed swelling of the basal region and normal growth of the apical region, as expected if polarity establishment and maintenance are functioning (Figure 4, Table 4). When the mutants were incubated at permissive temperature for 10 hr and then shifted to restrictive temperature, the effects on them were more subtle than those seen on the nonpolar *swo* mutants (Figure 5, Table 4). *SwoB* gave the most dramatic result, with slight swelling and an abnormally high number of branches. This may indicate that *swoB* plays a role in branch formation. *SwoE* showed slight basal and apical swelling, while neither *swoG* nor *swoH* showed obvious abnormalities. The less-pronounced effect of the shift from permissive to restrictive temperature may indicate that the events that require *swoG* and *H* have been successfully completed before the shift to restrictive temperature. That is, they take place early in hyphal morphogenesis. However, in view of the phenotypes of *swoG* and *H* at restrictive temperature and their behavior in downshift experiments, it seems more likely that their effects are simply not seen within the 2-hr time period of growth at restrictive temperature.

Model for the roles of the *swo* genes: We propose the following model for polarity establishment, polarity maintenance, and hyphal morphogenesis in *A. nidulans* (Figure 7):

1. Close to the second nuclear division, the expanding conidium switches from isotropic to polar growth (Figure 3). This switch requires establishment of an axis of polarity that anticipates the location of germ tube emergence. The products of *swoC*, *D*, and *F* are all required to establish polarity based on the inability of these mutants to make germ tubes when released from the restrictive temperature in downshift experiments (Figure 4, Table 4). The *swoC* and *D* gene products are not required for germ tube emergence or apical extension based on the normal hyphae made by these mutants at restrictive temperature in upshift experiments (Figures 5 and 6, Table 4). It should be noted that subsequent nuclear divisions are not dependent upon the establishment of polarity, *i.e.*, there is no "polarity checkpoint" that affects nuclear division. If that were the case, the *swo* mutants would arrest with only two nuclei (Figure 2, Table 3).
2. After polarity is established, a separate gene product

is needed to maintain the axis of polarity so that the germ tube emerges. The product of the *swoA* gene is required for polarity maintenance, but not its establishment, based on the ability of the *swoA* mutant to make germ tubes rapidly upon release from restrictive temperature in downshift experiments and on its inability to maintain polarity in upshift experiments (Figures 4 and 5, Table 4). Because of its association with apically extending tip cells, we speculate that the *swoA* gene product may interact with actin and/or the Spitzenkörper. The swoA protein may also show similarities to the tea1p or pom1p proteins that appear to organize polarity by marking the ends of *S. pombe* cells (Mata and Nurse 1997; Bahler and Pringle 1998). Future work will address these issues.

3. The polarity maintenance gene product required for germ tube emergence is also required for apical extension to continue. Even well after polarity has been established, the *swoA* mutant is not able to extend apically at restrictive temperature (Figure 6, Table 4). The *swoF* product also appears to play some role in polarity maintenance, in addition to its role in polarity establishment, on the basis of impaired apical extension in upshift experiments (Figures 5 and 6, Table 4).
4. Eventually the elongating hypha forms the branches and septa seen in mature fungal colonies. The products of *swoB*, *E*, *G*, and *H* all appear to be involved in establishing proper hyphal morphogenesis with postulated roles, including cell wall synthesis (*swoB*) and branch formation (*swoG*). Future work will examine these later events in hyphal morphogenesis more closely.

We thank John E. Hamer for generously providing the temperature-sensitive mutant collection, Jered Brown for technical assistance, and Debbie Haas for critical reading of the manuscript. This work was supported by a grant to M.M. from the Division of Energy Biosciences, U.S. Department of Energy. P.J.W. was supported by a predoctoral training grant in molecular and cellular mycology from the National Institutes of Health.

LITERATURE CITED

Bahler, J., and J. R. Pringle, 1998 Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis. Genes Dev. **12:** 1356–1370.

Bartnicki-Garcia, S., and E. Lippman, 1969 Fungal morphogenesis: cell wall construction in *Mucor rouxii.* Science **165:** 302–304.

Borgia, P. T., and C. L. Dodge, 1992 Characterization of *Aspergillus nidulans* mutants deficient in cell wall chitin or glucan. J. Bacteriol. **174:** 377–383.

Borgia, P., Y. Miao and C. L. Dodge, 1996 The *orlA* gene from *Aspergillus nidulans* encodes a trehalose-6-phosphate phosphatase necessary for normal growth and chitin synthesis at elevated temperatures. Mol. Microbiol. **20:** 1287–1296.

Bruno, K. S., R. Aramayo, P. F. Minke, R. L. Metzenberg and M. Plamann, 1996 Loss of growth polarity and mislocalization of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. EMBO J. **15:** 5772–5782.

Bull, A., 1970 Chemical composition of wild-type and mutant *Aspergillus nidulans* cell walls. The nature of polysaccharide and melanin constituents. J. Gen. Microbiol. **63:** 75–94.

Elorza, M. V., H. Rico and R. Senandreu, 1983 Calcofluor white alters the assembly of chitin fibrils in *Saccharomyces cerevisiae* and *Candida albicans* cells. J. Gen. Microbiol. **129:** 1577–1582.

Harris, S. D., 1997 The duplication cycle in *Aspergillus nidulans.* Fungal Genet. Biol. **22:** 1–12.

Harris, S. D., J. L. Morrell and J. E. Hamer, 1994 Identification and characterization of *Aspergillus nidulans* mutants defective in cytokinesis. Genetics **136:** 517–532.

Harris, S. D., L. Hamer, K. E. Sharpless and J. E. Hamer, 1997 The *Aspergillus nidulans sepA* gene encodes an FH1/2 protein required for cytokinesis and the maintenance of cellular polarity. EMBO J. **16:** 3474–3483.

Heath, I. B., 1990 The roles of actin in tip growth of fungi. Int. Rev. Cytol. **123:** 95–127.

Kafer, E., 1977 Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. **19:** 33–131.

Kaminskyj, S. W., and J. E. Hamer, 1998 *hyp* loci control cell pattern formation in the vegetative mycelium of *Aspergillus nidulans.* Genetics **148:** 669–680.

Kruger, M., and R. Fischer, 1998 Integrity of a Zn finger-like domain in SamB is crucial for morphogenesis in ascomycetous fungi. EMBO J. **17:** 204–214.

Lew, D. J., and S. I. Reed, 1995 Cell cycle control of morphogenesis in budding yeast. Curr. Opin. Genet. Dev. **5:** 17–23.

Livingston, L. R., 1969 Locus-specific changes in cell wall composition characteristic of osmotic mutants of *Neurospora crassa.* J. Bacteriol. **99:** 85–90.

Maeda, H., and N. Ishida, 1967 Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J. Biochem. **62:** 276–278.

Marks, J., and J. S. Hyams, 1985 Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe.* Eur. J. Cell Biol. **39:** 27–32.

Mata, J., and P. Nurse, 1997 tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell. Cell **89:** 939–949.

McGoldrick, C. A., C. Gruver and G. S. May, 1995 *myoA* of *Aspergillus nidulans* encodes an essential myosin I required for secretion and polarized growth. J. Cell Biol. **128:** 577–587.

Mitchison, J. M., and P. Nurse, 1985 Growth in cell length in the fission yeast *Schizosaccharomyces pombe.* J. Cell Sci. **75:** 357–376.

Reynaga-Pena, C. G., G. Gierz and S. Bartnicki-Garcia, 1997 Analysis of the role of the Spitzenkörper in fungal morphogenesis by computer simulation of apical branching in *Aspergillus niger.* Proc. Natl. Acad. Sci. USA **94:** 9096–9101.

Roemer, T., L. G. Vallier and M. Snyder, 1996 Selection of polarized growth sites in yeast. Trends Cell Biol. **6:** 434–441.

Roncero, C., M. H. Valdivieso, J. C. Ribas and A. Duran, 1988 Isolation and characterization of *Saccharomyces cerevisiae* mutants reistant to Calcofluor White. J. Bacteriol. **170:** 1950–1954.

Seiler, S., F. E. Nargang, G. Steinberg and M. Schliwa, 1997 Kinesin is essential for cell morphogenesis and polarized secretion in *Neurospora crassa.* EMBO J. **16:** 3025–3034.

Ste-Marie, I., S. Senechal, M. Boushira, S. Garzon, H. Strykowski *et al.*, 1990 Production and characterization of monoclonal antibodies to cell wall antigens of *Aspergillus fumigatus.* Infect. Immun. **58:** 2105–2114.

Communicating editor: P. J. Pukkila