The so Locus Is Required for Vegetative Cell Fusion and Postfertilization Events in Neurospora crassa

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The process of cell fusion is a basic developmental feature found in most eukaryotic organisms. In filamentous fungi, cell fusion events play an important role during both vegetative growth and sexual reproduction. We employ the model organism Neurospora crassa to dissect the mechanisms of cell fusion and cell-cell communication involved in fusion processes. In this study, we characterized a mutant with a mutation in the gene so, which exhibits defects in cell fusion. The so mutant has a pleiotropic phenotype, including shortened aerial hyphae, an altered conidiation pattern, and female sterility. Using light microscopy and heterokaryon tests, the so mutant was shown to possess defects in germling and hyphal fusion. Although so produces conidial anastomosis tubes, so germlings did not home toward wild-type germlings nor were wild-type germlings attracted to so germlings. We employed a trichogyne attraction and fusion assay to determine whether the female sterility of the so mutant is caused by impaired communication or fusion failure between mating partners. so showed no defects in attraction or fusion between mating partners, indicating that so is specific for vegetative hyphal fusion and/or associated communication events. The so gene encodes a protein of unknown function, but which contains a WW domain; WW domains are predicted to be involved in protein-protein interactions. Database searches showed that so was conserved in the genomes of filamentous ascomycete fungi but was absent in ascomycete yeast and basidiomycete species.

The mycelial colony of filamentous fungi, such as Neurospora crassa, consists of a network of interconnected, multinucleate hyphae. Hyphae grow by tip extension and undergo branching and hyphal fusion (anastomosis) (4). The formation of mycelial networks by anastomosis presumably increases cytoplasmic flow and is important for the distribution of nutrients and signals within the colony (24, 46). While tip growth and branching have been the subject of numerous studies (13, 18, 19, 56), the process of anastomosis is only poorly understood (14, 15).

Live-cell imaging of anastomosis in vegetative colonies of N. crassa has shown it to be a complex signaling and cell biological process (20). Hyphal branches in the sub-peripheral regions of a colony show chemotropic interactions culminating in fusion (20), while hyphae at the periphery of a colony generally avoid contact. Anastomosis in filamentous fungi is comparable to fusion events between cells of a single organism, such as during organogenesis in metazoans (52, 54), as well as fusion events between cells from different individuals, such as mating in Saccharomyces cerevisiae (9, 59) and fertilization in animals.

In fungi, both vegetative and sexual fusions require extracellular communication between fusion partners, resulting in directed growth. After physical contact, the cell wall must be broken down between fusion cells, followed by plasma membrane fusion and pore formation (14, 15, 20, 59). In N. crassa, a number of genes have been characterized that are required for hyphal fusion. The ham-2 locus encodes a putative transmembrane protein necessary for hyphal fusion (62). Recently, an ortholog of ham-2 in S. cerevisiae, FAR11, was shown to be required for pheromone-initiated cell cycle arrest during mating (26). Loci that encode components of a mitogen-activated protein kinase (MAPK) signal transduction pathway are also required for vegetative hyphal fusion in filamentous fungi (39, 57). A MAPK, MAK-2, and a MAPK kinase kinase (MEKK), NRC-1, have been shown to be essential for anastomosis in N. crassa (39, 47). The respective orthologs of the MAPK and MEKK in S. cerevisiae (STE11 and FUS3) are members of a MAPK pathway that is activated in response to pheromone during mating (5, 9, 10). Although mating and hyphal fusion show some similarities, chemotropic interactions in these two processes are different; N. crassa strains that carry a mutation in the putative mating pheromone receptor gene, pre-1, fail to mate, but are unaffected in vegetative cell fusion (27).

In addition to anastomosis within a mature fungal colony, cell fusion events are associated with other developmental stages in filamentous fungi. Germinating conidia can produce specialized hyphae called conidial anastomosis tubes (CATs), which fuse with each other (29, 47, 48) to establish a hyphal network that rapidly develops into a colony. Fusion events are also required during sexual reproduction in many filamentous ascomycete species. In N. crassa, specialized female hyphae (trichogyynes) emerge from ascogonia and are attracted to and fuse with a male mating partner of the opposite mating type (microconidium, conidium, or a vegetative hypha) (3, 27). Following fusion, opposite mating-type nuclei proliferate within the ascogonium and subsequently pair to form ascogenous
hyphae. Prior to karyogamy, opposite mating-type nuclei are compartmentalized in the penultimate cell of the ascogenous hypha (crossover). Cell fusion subsequently occurs in the crossover between cells that subtend the developing ascus (45).

In this study, we characterized the so (soft) mutant of *N. crassa*. The so mutant shows a pleiotropic phenotype, including shortened aerial hyphae, an altered condiation pattern, and female sterility. We determined that so is allelic to *ham-1*; mutations at *ham-1* result in strains that fail to form heterokaryons (61). We show that so mutants fail to undergo both hyphal and germling fusion. However, so germlings form CATs, although so CATs fail to show chemotropic interactions with wild-type germlings. Although defects in vegetative fusion were apparent in so mutants, fusion events associated with fertilization were normal. The so gene was cloned by complementation. Database searches using BLAST revealed that so orthologs are present only in the genomes of filamentous ascomycete fungi, although predicted proteins with that similarity to the carboxy-terminal portion of so are present in other fungi.

**MATERIALS AND METHODS**

*N. crassa* strains and growth media. Strains used in this study are listed in Table 1. Strains were grown on Vogel's minimal medium (MM) (55). For strains carrying auxotrophic markers, required supplements were added to the medium. Crosses were performed on Westergaard's medium (58). If strains carrying auxotrophic markers were used as females, one-tenth of the normally required concentration of the supplement was added to the mating medium. Alternatively, a heterokaryon between strain FGSC 4564 (Fungal Genetics Stock Center) (Table 1) and the auxotrophic strain was used as a female in crosses (40). FGSC 4564 carries a mutation (Δm1) in its mating-type locus. This mutant shows normal vegetative growth and forms female sexual reproductive structures (protophoretica). A heterokaryon between Δm1 and an auxotrophic strain will form protoperithecia on mating medium lacking supplements. Following fertilization by the opposite mating type, only nuclei from the male parent and the auxotrophic strain will contribute to the cross; Δm1 nuclei cannot participate in a cross. To test the mating type of strains, they were crossed with mating-type fl a (FGSC 4347) and fl A (FGSC 4317) tester strains (Table 1).

**Heterokaryon tests and growth rate measurements.** To form a heterokaryon between two strains carrying different auxotrophic markers, conidia from seven- to ten-day-old cultures of the respective strains were mixed and applied as spots on Vogel's MM without supplements. Alternatively, small pieces of mycella from the respective strains were inoculated in direct physical contact on MM. To form heterokaryons with FGSC 4564 for crosses, the two strains were directly inoculated on Westergaard's medium. Heterokaryons between so and *ham-1* strains were obtained by protoplast fusion. Approximately 10^7* *M. macrocystidium* were spread on a cellophane layer placed on Vogel's MM agar containing appropriate supplements. Conidia were germinated for 4 h at 30°C. Two cellophane layers per strain were transferred into a petri dish containing 15 ml of 1.0 M MgSO_4 with 90 mg lysing enzyme (Sigma), 90 mg β-o-glucanase (Interspec Products), and 3 mg Driselase (Sigma). After 2 h of incubation at 30°C with slight shaking, the suspension was filtered through six layers of cheesecloth. The protoplasts were concentrated by centrifugation (3,000 rpm) and washed once with ice-cold 1.0 M MgSO_4 and once with ice-cold STC (1.0 M sorbitol, 50 mM Tris, pH 8.0, 50 mM CaCl_2). The protoplasts were resuspended in 300 μl STC, 100 μl of each protoplast suspension was mixed, and an aliquot of 50 μl PTC (40% polyethylene glycol [PEG] 4000, 50 mM Tris, pH 8.0, 50 mM CaCl_2) was added. After 25 min of incubation at room temperature, 6 ml PTC was added. This suspension was mixed with 60 ml of regeneration agar (49) and poured into three petri dishes containing MM. Prototrophic heterokaryons were isolated after approximately three days of incubation at 30°C.

To quantify the ability of strains to form heterokaryons (wild type [WT] + WT, WT + so, and so + so), quantitative heterokaryon tests were performed (62). Conidial suspensions (1 × 10^7 conidia) of tester strains IK08-7a (pyr-4 a) or R9-01 (so; pyr-4 a) were mixed with 100,000, 10,000, 5,000, or 1,000 conidia of 2041-11 (nic-3; inl Δm33) or 2041-18 (so; inl Δm33). Conidial mixtures were plated on BDES medium (7), which causes *N. crassa* to grow as compact colonies. After 2 days of incubation at 30°C, the number of heterokaryotic colonies per plate was counted. Viability of conidia was determined by plating 300 and 3,000 conidia from each strain individually on BDES medium containing the required supplements. All experiments were performed in triplicate. The concentration of conidial suspensions was determined by counting with a hemacymeter.

Growth rates were determined by growing strains in race tubes. Race tubes (approximately 45 cm in length) were filled with 25 ml of MM agar. For auxotrophic strains, required supplements were added. The tubes were inoculated at room temperature (22°C), and growth was measured at 24-h intervals for eight days.

**Nucleic acid isolation and Southern hybridization.** Genomic DNA was isolated according to Lee and Taylor (32). Total RNA was isolated using a RNA isolation kit (Promega RNAs) according to the manufacturer's instructions. Southern hybridization was performed as described by Sambrook et al. (50).

**Cloning strategy.** Linkage group I cosmids constructed in vector pLoriosth (conferring hygromycin resistance) were obtained from the FGSC (University of Missouri; http://ww.fgsc.net/). Cosmid DNA was isolated using a Plasmid Midi prep kit (QIAGEN). Conidia of so strain R9-01 were transformed by electroporation following the description in reference 36, using 1.5-kV and 1-mm-gap cells (R. L. Metzenberg and K. Black, personal communication). After identification of a cosmid that complemented the so mutant phenotype, each open reading frame (ORF) predicted in this cosmid was cloned into pCB1004 (conferring hygromycin resistance) (6) and subsequently used to transform so (R9-01) and *ham-1* (R9-02) strains. Predictions about the number and positions of ORFs were obtained from the N. crassa genome project of the Broad Institute (http://www.broad.mit.edu/annotation/fungi/neurospora/) and *Neurospora* Sequencing Project at the Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de/genre/neurospora/).

**Light and fluorescence microscopy.** For light or fluorescence microscopy, hyphae were observed on agar blocks containing either Vogel's MM or 1/10 Vogel's MM or on cellophane removed from inoculated agar plates. Mycelia and conidia were examined using bright field or differential interference contrast optics with a Zeiss Axioskop II microscope or a Nikon TE 2000E inverted microscope. Fluorescence microscopy was performed using a Zeiss Axioskop II with the Chroma Standard Filter set 31019 (Chroma Technology Corp) filter block.

Hyphae were prepared and stained with FM4-64 as described in Hickey et al. (20) and imaged by confocal microscopy as described by Pandey et al. (39).

**TABLE 1. Strain origin and genotype**

| Strain | Genotype | Origin |
|--------|----------|--------|
| FGSC 988 | a | FGSC* |
| FGSC 2489 | A | FGSC |
| FGSC 508 | so A | FGSC |
| FGSC 542 | so a | FGSC |
| AF-H4 | so; his-3-A | This study |
| R9-01 | so; pyr-4 a | This study |
| R9-02 | ham-1; nic-1; al-1 A | This study |
| R1-19 | cyh-1; pyr-4 A | Gift from R. L. Metzenberg |
| IK08-7a | pyr-4 a | Gift from I. Kaneko |
| 2042-11 | inl; nic-3 aΔm33 | This study |
| 2041-18 | so; inl aΔm33 | This study |
| R11-03 | his-3::H1-GFP A | Progeny from H1-GFP strain |
| R12-60 | his-3::H1-GFP a | Progeny from H1-GFP strain |
| R13-28 | his-3::H1-GFP so a | This study |
| R13-29 | his-3::H1-GFP so A | This study |
| FGSC 4564 | ad-3B cyh-1 aΔm1 | FGSC |
| FGSC 2225 (MV) | A | FGSC |
| 1c | | |
| R16-06 | arg-13 so aro-8 a | This study |
| FGSC 4317 | fla A | FGSC |
| FGSC 4347 | fla a | FGSC |

* FGSC, Fungal Genetics Stock Center.
Hyphae stained with 0.1 μM Calcofluor white M2R were prepared and imaged by confocal microscopy as described by Hickey et al. (21). To observe germinating events, 3 × 10⁶ conidia were spread on a pipette tip directly onto cellophane layered onto 1/10 Vogel’s MM plates. Plates were incubated at 25°C and analyzed at different times following inoculation. Alternatively, 1 × 10⁶ conidia were germinated in 1 ml 1/10 liquid Vogel’s MM without shaking at 25°C. At different times, samples were taken and analyzed by microscopy.

Homing and germling fusion were assessed between (so + so), (so + wild-type) and (wild-type + wild-type) combinations 3 to 4 h after inoculation into liquid Vogel’s MM in an eight-well slide culture chamber (47) and visualized by combining differential interference contrast and fluorescence imaging using a Nikon TE2000E inverted microscope. One thousand germlings were examined for each strain combination. The so strains used were FGSC 508 and FGSC 542; the wild-type strains used were R11-03 and R12-60.

Optical tweezers assay. Growth interactions between CATs of the so mutant FGSC 542, of the so mutant FGSC 542 and the wild-type strain R12-60, and of the wild-type strain R12-60 alone were analyzed in liquid Vogel’s MM after manipulation using the optical tweezers technique described by Roca et al. (47). Germlings were moved relative to each other, and the reorientation (if any) of the CATs toward each other was monitored with time-lapse imaging. At least 10 germlings were manipulated for each strain combination.

Trichogyne assay. Trichogyne assays were performed as described by Bistis (3). The centers of plates containing 2% water agar (Bacto agar; Difco) were inoculated with a small piece of mycelium. The plates were incubated for 2 days in a humidity chamber and then transferred to the bench top for 5 more days. After 7 days of incubation, thin slices (approximately 5 by 5 by 1 mm) of 2% water agar were placed on top of groups of one to five protoperithecia. Agar slices were obtained by pouring 6 ml of molten agar into a petri dish and cutting the solidified layer with an inoculation lancet (Karl Roth GmbH&Co, Karlsruhe, Germany). A water droplet containing 5 to 100 microconidia was applied as a spot on top of the agar slice. After 20 to 24 and 40 to 44 h of incubation at room temperature, the plates were evaluated by bright-field and fluorescent microscopy. Microconidia were obtained following the protocol described by Ebbole and Sachs (8).

Computational analysis. Database searches via BLAST (1) were conducted at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/BLAST). Searches for conserved domains were conducted using the NCBI Conserved Domain Search (35) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). For protein localization predictions, PSORTII (23) (http://www.psort.org/) was used. Signal peptide predictions were conducted using SignalP (38) (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane prediction programs used were TMHMM (31) (http://www.cbs.dtu.dk/services/TMHMM/) and SOSUI (22) (http://sosui.proteome.bio.tuat.ac.jp).

RESULTS

ham-1 and so are allelic. Wilson and Dempsey (61) published a brief report on the first hyphal fusion mutant in N. crassa, which was called ham-1 (hyphal anastomosis mutant). We mapped ham-1 to linkage group 1 between arg-13 and aro-8. Earlier studies mapped another mutation called so to the same interval (42). The so and ham-1 mutants showed similar phenotypes, including shortened aerial hyphae, relatively sparse conidiation (Fig. 1A, B) and female sterility (41, 43). These observations suggested that so and ham-1 might be allelic. To test this hypothesis, we used protoplast fusion to force heterokaryons between auxotrophic ham-1 (R9-02) and so (AF-H4) strains (Table 1). The (ham-1 + so) heterokaryons displayed a phenotype identical to that of the ham-1 and so mutants individually, indicating allelism. This result was confirmed by complementation of ham-1 by introduction of the so gene (see below).

The original ham-1 mutant was identified during analysis of a cross between an Oak Ridge-St. Lawrence strain and a Rockefeller-Lindgren (RL) strain (60, 61). Because of the mixed genetic background of the ham-1 mutant, we focused on phenotypic and molecular characterization of so strains, which are of laboratory standard background (Oak Ridge).

so is a hyphal fusion mutant. Since so and ham-1 were allelic, we expected the so strain to be a hyphal fusion mutant. Microscopic observations revealed that the so mutant was defective in forming hyphal anastomoses in the interior of a colony (Fig. 2D, F). Although hyphal branches within subperipheral regions of a wild-type colony showed attraction and underwent hyphal fusion (20) (Fig. 2C, E), fusion bridges of this type were not observed within a so colony. Even in cases of physical contact between two so hyphae, cell wall breakdown and cytoplasmic mixing were not observed (Fig. 2F). As in wild-type strains, so hyphae in the colony periphery grew outward and avoided contact with each other, although the number of leading hyphae at the periphery of a so colony was less than in wild-type strains (compare Fig. 2A and 2B).

Conidia and conidial germlings of N. crassa fuse during early stages of colony establishment (29, 39, 47). To determine whether germling fusion occurs in so mutants, conidia from so (FGSC 508 and FGSC 542; Table 1) and wild-type (FGSC 988 and FGSC 2489; Table 1) strains were germinated on solid medium or in liquid culture (see Material and Methods) and analyzed by microscopy. Under both growth conditions, conidia of wild-type strains germinated ~1 to 2 h after inoculation (25°C). Fused germlings were detectable after 4 to 6 h (Fig. 3A). Although conidia from so strains germinated within the same time frame as wild-type conidia, fused conidia or germlings were never observed on solid medium or in liquid culture (Fig. 3B).

Since so germlings lacked fusion, we evaluated whether so was able to form conidial anastomosis tubes (CATs). CATs are significantly thinner than germ tubes and can be distinguished from germ tubes by measuring their widths (47). Germ tubes of R12-60 (Table 1) had an average width of 4.79 ± 0.47 μm, while the average for CATs was only 2.26 ± 0.31 μm. Germinating conidia of the so strain (FGSC 542) produced germ tubes which were 4.75 ± 0.45 μm wide and also formed thinner hyphae which were 2.72 ± 0.39 μm in width, indicating that so

FIG. 1. Macroscopic phenotype of the so mutant. The so (soft) mutant exhibits short aerial hyphae and an altered conidiation pattern. (A) Agar flask cultures. Left, wild type (FGSC 2489); right, so mutant (FGSC 542). (B) Agar plate cultures. Top, wild-type strain (FGSC 2489); bottom, so mutant (FGSC 542).
Probable forms CATs (Fig. 3C; white arrow). However, the relative number of so conidia producing CATs was significantly lower as compared with wild type. While 36% ± 3% of R12-60 conidia formed CATs, only 10% ± 2% of the so strain (FGSC 542) developed these structures.

so germlings fail to attract or respond to wild-type germlings. During anastomosis and germling fusion, chemotropic interactions require a signal indicating the presence of a fusion partner and the machinery to perceive and process this signal. To test whether the so mutation affected signal production or perception of the chemotropic signal, we confronted so and wild-type conidia. If so was affected in the formation of the fusion signal, we expected that homing of wild-type germlings toward so conidia would not occur. If so is required for perception and processing of the signal, we expected to observe homing of the wild type toward the so mutant, but the so mutant would be incapable of responding to the presence of wild-type conidia. To distinguish so and wild-type conidia, we used a wild-type strain (R12-60) expressing nucleus-targeted H1-green fluorescent protein (GFP) (12). The same number of conidia of R12-60 and so conidia (FGSC 542) (total concentration of 10⁶ conidia per ml) were mixed in liquid Vogel’s medium (see Materials and Methods). After 4 h, 2,000 conidia/conidial germlings were analyzed by light microscopy. Homing and fusion were observed only between conidia of R12-60, while no reactions between two so conidia or between a so conidium and a wild-type conidium were ever observed (Fig. 3C).

To study the interaction between conidial germlings in more detail, we used optical tweezers in a CAT homing assay (47). Here adjacent so germlings (FGSC 542) or so germlings adjacent to wild-type germlings (R12-60) were moved <5 μm apart, and subsequent growth reorientation and fusion were monitored. Using this assay, homing and fusion between CATs of wild-type conidia were consistently shown (Fig. 4A, n = 5) (also see reference 47). However, the CATs of wild-type and so conidia showed no evidence of attraction and growth toward each other (n = 15) (Fig. 4B). Some drifting of germlings was observed in this assay: WT germling confrontations resulted in reorientation and homing, although so-so or so-WT germling interactions did not. These data suggest that so is defective in both the production of a chemotropic signal as well as in its perception. We observed that the width of CATs sometimes increased after optical tweezer micromanipulation (Fig. 4A). The effect does not seem to be due to the tweezer manipulation itself because it often occurs in the wild type in the absence of manipulation.

In addition to the analysis of hyphal fusion by microscopy, we investigated the fusion defect in so by quantitative heterokaryon tests using strains with complementing auxotrophic markers (see Materials and Methods). In this assay, a known number of reference conidia from one auxotrophic strain were spread onto a number of plates, while successive dilutions of the test conidia from the second auxotrophic strain were subsequently spread onto the same plates. Successful fusion events are evident by the growth of discrete heterokaryotic colonies on the plates. When the frequency of heterokaryon formation between two wild-type strains [IK08-7a (pyr-4 a) + 2042-11 (int; nic-3 aⁿʷ³)] was compared to the frequency of heterokaryon formation between two so strains [R9-01 (so; pyr-4 a) + 2041-18> (so; int aⁿʷ³)] or a so strain and a wild-type strain (R9-01 + 042-11; 2041-18 + IK08-7a), the capacity of so strains to form a heterokaryon was reduced at least 300-fold.

Maximal hyphal extension is not drastically impaired by mutations in so. Wilson and Dempsey described the ham-1 mutant as slow growing (61). Because of the mixed genetic background of the ham-1 mutant, we chose to measure the growth rate of the so mutants (FGSC 508 and FGSC 542; Table 1) as compared with wild-type strains (FGSC 988 and FGSC 2489; Table 1), which are all of standard laboratory background. Wild-type strains FGSC 988 and FGSC 2489 had growth rates of 6.7 (± 0.3) and 6.4 (± 0.5) cm/day, respectively. The so strains FGSC 508 and FGSC 542 showed growth rates of 4.6 (± 0.9) and 5.4 (± 0.4) cm/day, respectively. Growth measurements of two pyr-4 strains, IK08-7a (pyr-4 a) and R9-01 (so; pyr-4 a) showed growth rates of 6.1 (± 0.5) and 5.8 (± 0.3) cm/day, respectively. Thus, maximal colony extension was only
slightly reduced in so strains as compared to wild-type strains. However, wild-type strains have very regular and dense colony margins which consist of hyphae that exhibit similar linear extension rates (Fig. 2A). In contrast, the colony margins of so were very irregular (Fig. 2B). In so colonies, only a few hyphae at the periphery of the colony showed maximal extension rates, while the growth of other hyphae lagged.

The so mutant is blocked in sexual development. The so mutant was reported to be female sterile (43), although the basis of the female sterility was unclear. We therefore com-

FIG. 3. The so mutant shows defects in germling fusion. (A) Fusion (indicated by arrow) between germinating conidia of wild-type FGSC 2489 in stationary liquid culture. (B) so (FGSC 542) conidia in stationary liquid culture germinate at a comparable time to that of a wild-type strain but do not show germling fusion events. If germlings appeared to have physical contact (arrow), no cell fusion events were observed. The inset shows the area of physical contact at a higher magnification. (C) Confrontation between so (FGSC 542) and a wild-type (Wt) strain (R12-60) on solid medium. The wild-type strain is labeled by H1-GFP (H1) (white nuclei). While homing and fusion between wild-type conidia were observed, (black arrow), a reaction between so conidia or so and wild-type conidia did not occur. CATs, however, did form in so strains (white arrow) but were formed at a reduced frequency compared to that in a wild-type strain. Bars = 10 μm.

FIG. 4. so conidial anastomosis tubes do not home and fuse. (A) The CATs of two wild-type (R12-60) macroconidia home toward each other (0 min). The lower germling was manipulated by optical tweezers and moved slightly to the right (5 min). The two CATs subsequently homed back toward each other and fused (59 min). Note that the CATs increased in width after manipulation. (B) CATs from so (FGSC 542) and wild-type (R12-60) macroconidia, which were orientated toward each other via manipulation by optical tweezers, were followed in a time course. The growth of the wild-type and so conidia toward each other was not due to homing, and when the CATs touched they did not fuse. Note that the germlings showed some drifting and rotation but fail to show chemotropic interactions. The wild-type germlings were distinguished from the so germlings by labeling with nucleus-targeted H1-GFP (data not shown). Bar = 5 μm.
pared the development of so with a wild-type strain at different steps of the sexual cycle. The so strains, FGSC 508 and FGSC 542, and the wild-type strains, FGSC 988 and FGSC 2489, were inoculated onto mating plates and incubated at room temperature. After 7 days, all strains had formed protoperithecia, the female reproductive structures. The microscopic appearance of so protoperithecia was comparable to that of wild-type strains. Thus, the female fertility defect reported in the so mutant was not due to a failure to initiate protoperithecial development and maturation.

Entry into sexual reproduction requires cell fusion between a filamentous trichogyne, which originates from the protoperithecum, and a male cell of the opposite mating type (macroconidia, microconidia, or vegetative hypha) (3). Trichogynes show chemotropic attraction and growth toward a male cell of the opposite mating type but show no such behavior toward male cells of an identical mating type (3, 27). Since the so mutation affected homing and fusion during vegetative growth, we tested whether so trichogynes were defective by conducting a trichogyne attraction assay (3). Microconidia of either the same or different mating type (R11-03, R12-60) were placed on top of wild-type (FGSC 988, FGSC 2489) and so (FGSC 508, FGSC 542) protoperithecia. Attraction of trichogynes was evaluated by microscopy 22 and 44 h later. Out of 54 wild-type mat a trichogynes (FGSC 988), 48 (89%) showed attraction to and physical contact with mat A conidia (R11-03). Out of 24 mat A trichogynes (FGSC 2489), 23 (96%) showed attraction to and contact with mat A conidia (R12-60) (Fig. 5A). Similar to trichogynes of wild-type strains, so trichogynes exhibited attraction to conidia of the opposite mating type (Fig. 5B). Out of 34 so a (FGSC 542) trichogynes, 30 (88%) showed attraction and physical contact with mat A conidia (R11-03). Fifty-four out of 61 (89%) so mat A trichogynes (FGSC 508) were attracted to mat a conidia (R12-60). Attraction of trichogynes to conidia of the same mating type was not observed for wild-type or so trichogynes.

After contact between a trichogyne and a male cell, the transfer of the male nucleus into the protoperithecum requires cell fusion. Although the so trichogynes showed normal attraction and contact with conidia of the opposite mating type, it was possible that the cell fusion event between a trichogyne and conidium was blocked. We reasoned that if so trichogynes are capable of this fusion event, disappearance of the nucleus from the male cell should be observed after successful fusion. To test this hypothesis, we used microconidia from strains expressing nucleus-targeted H1-GFP (R11-03, R12-60), which could be visualized as male crossing partners by fluorescence microscopy; microconidia of N. crassa typically contain only a single nucleus (8, 34). In wild-type crosses (FGSC 988 × R11-03; FGSC 2489 × R12-60), the nuclear GFP signal in the H1-GFP microconidium disappeared upon contact between the trichogyne and the microconidium (Fig. 5A). Since the disappearance of the GFP nuclear signal was never observed without contact between an H1-GFP microconidium and a trichogyne, loss of the GFP signal was considered to be a consequence of a fusion event. Whether the loss of the GFP signal is due to the movement of the male nucleus from the trichogyne into the protoperithecum, dilution of GFP-labeled histone or some kind of silencing event is unknown. Similar to wild-type crosses, loss of GFP signal in the microconidia was observed in crosses using so (FGSC 508, FGSC 542) as a female and H1-GFP microconidia (R11-03, R12-60) (Fig. 5B). Homozygous so crosses using so (H1-GFP) microconidia (R13-28, R13-29) as a male showed identical behavior (data not shown). These results indicate that the so mutant is not defective for trichogyne-conidium attraction or fusion events.

Although protoperithecial formation, trichogyne attraction, and trichogyne-conidium fusion were not defective in so mutants, fertilized so protoperithecia failed to develop further. Fertilized so protoperithecia did not enlarge or melanize and asc or ascospores were not produced. These observations indicate that the block in development of so protoperithecia following fertilization by either so or WT conidia occurred early during sexual development. To further investigate the defect causing the female infertility of so crosses, we employed a strain carrying the am1 mating-type allele (17). The am1 mutant, which contains a loss-of-function mutation at the mat locus (53), forms female reproductive structures, but cannot participate in a sexual cross either as a male or a female (40). Earlier studies investigating homozygous crosses between a septation-defective mutant (cw1) with a (cw1 + am1) heterokaryon indicated that am1 nuclei are not present or are not active in ascogenous hyphae (44). We therefore used a (so + am1) heterokaryon to evaluate the influence of the so mutation in crozier and ascus development in so homzygous crosses.

A heterokaryon between a so strain (R9-01) and am1 (FGSC 4564) was grown on mating medium. After 7 days, protoperithecia were fertilized with macroconidia from FGSC 508 (so A). Perithecial development of the [so A × (so a + am1)] was indistinguishable from that of wild-type crosses, including asc and ascospore development, indicating that the am1 strain complemented the female fertility defect in the so mutant. Cell fusion within the croizer was normal (N. B. Raju, personal communication), indicating that so is not required for cell fusion events associated with croizer development, nor for asc and ascospore development. These observations suggest that so is required in the interval between trichogyne-conidium fusion and establishment of ascogenous hyphae.

Cloning of so. To clone so, we used both genetic data and restriction fragment length polymorphism (RFLP) analysis. Earlier studies mapped so between arg-13 and aro-8 on linkage group I (LGI) (42). The respective recombination rates between arg-13 and so were 2% to 12%, while the recombination rate between so and aro-8 was 7% to 11%, depending on the parents of the cross. To bracket so between arg-13 and aro-8, we employed RFLP analysis. Numerous RFLP differences occur between the standard laboratory strain (Oak Ridge background) and a Mauriceville strain, which had been used previously to develop a RFLP map for N. crassa (37). The Oak Ridge strain (arg-13 so aro-8 a) was crossed with the Mauriceville wild-type strain, FGSC 2225; progeny were selected for recombination between arg-13 and aro-8 and subsequently scored for the so phenotype. Genomic DNA from the parents (arg-13 so aro-8 a and FGSC 2225) and 20 progeny showing recombination between arg-13 and aro-8 were digested with XbaI or BamHI and probed with cosmids that spanned the arg-13 and aro-8 interval. Due to the ordering of the cosmid library on the N. crassa genome (http://www.broad.mit.edu/annotation/fungi/neurospora/), we used contiguous cosmids
FIG. 5. Trichogynes of so fuse with microconidia of the opposite mating type. Microconidia containing H1-GFP-tagged nuclei (R12-60 and R11-03) were placed on top of wild-type (FGSC 2489) (A) and so (FGSC 542) (B) protoperithecia of the opposite mating type, respectively. The interaction between trichogynes and microconidia was evaluated by microscopy 22 h (A1, B1) and 44 h (A2, B2) after inoculation. (A) Trichogynes from the WT strain (FGSC 2489) also show attraction to wild-type microconidia of the opposite mating type (R12-60) (black arrow). The GFP-labeled nuclei disappeared after contact between a wild-type trichogyne (FGSC 2489) and a conidium of the opposite mating type (R12-60) (white arrow). (B) Trichogynes from so (FGSC 542) also show attraction to wild-type microconidia of the opposite mating type (R11-03) (black arrow). Loss of nuclear GFP fluorescence was also observed (B1 versus B2; white arrow). Bar = 10 μm.
protoplast fusion experiments showed that sophae and conidiation pattern defect in the strain R9-01. Only NCU02794.1 complemented the aerial hyphae hygromycin resistance (6), and used to transform the predicted ORFs was cloned into pCB1004 plasmid, which contained a 2,105 bp of the 5′ half of the so ORF was amplified by PCR and cloned into pCB1004 (6). The resulting plasmid was transformed into R1-19 (cyh-1; pyr-4 A). Three different hygromycin-resistant transformants (T1 to T3) were crossed with FGSC 988, and 60 progeny were isolated from each cross. From the T1 cross, 9.6% of the progeny (P1), 18.6% from the T2 cross (P2) and 20.3% from the T3 cross (P3) showed a typical macroscopic so phenotype (Fig. 1A, B). The DNA sequence of the so allele from one of the so-like progeny from the T1 cross (P1-1), from two progeny from the T2 cross (P2-1 and P2-2) and from two progeny from the T3 cross (P3-1 and P3-2) was determined. The so sequence in every mutant showed numerous G-to-A or C-to-T mutations, the typical result of RIP mutations. These mutations led to the formation of stop codons in the 5′ portion of the so gene. Progeny P1-1 and P3-1 both have a G-to-A exchange at position 1769 creating a stop codon (W590Stop). The P2-1, P2-2, and P3-2 progeny have C-to-T exchanges creating stop codons at positions 547 (Q183Stop), 451 (Q183Stop), and 1243 (Q415Stop), respectively.

The capacity of the new so mutants (P1-1 and P3-2) to undergo germling and hyphal fusion was compared to that of so and wild-type strains. Conidia from P1-1 and P3-2 and FGSC 988 were spread onto Vogel’s minimal medium, and after 5 h of incubation at 30°C, the plates were analyzed for germling fusion by light microscopy. Although germling fusion was detectable at a high frequency in wild-type strains, germling fusion was not observed in conidia from P1-1 or P3-2. Hyphal anastomoses in one-day-old P1-1 and P3-2 colonies were also not observed. To assess the female fertility of P1-1 and P3-1, each strain was inoculated onto mating medium and crossed with conidia from FGSC 988 (mat a) and FGSC 2489 (mat A). Although both P1-1 and P3-1 formed protoperithecia, in no case was the development of fertile perithecia observed following fertilization with FGSC 988. These data show that the new so mutants showed an identical phenotype to that of the original so mutant, indicating that NCU02794.1 carries the so gene.

so is conserved in filamentous ascomycete species. An NCBI conserved domain search revealed that SO exhibits conserved features of a WW domain (aa 562 to 594). WW domains are characterized by two conserved tryptophan (W) residues spaced 20 to 22 amino acids apart (2, 33). This domain has been shown to mediate protein-protein interactions by recognizing proline-containing ligands. WW domains are found in

![FIG. 6. Cloning strategy for so. Contiguous cosmids in a region of ~130 kbp around the so locus on the right arm of LGI. Flanking markers arg-8 and arg-13 are indicated. H39A5 contains five predicted open reading frames (based on our analysis and http://www-genome.wi.mit.edu/annotation/fungi/neurospora). Boxes indicate ORFs (not drawn to scale) on contig 3.145 designated NCU02793.1 to NCU02797.1. Each ORF was cloned and transformed individually into a so mutant (R9-01) to assess complementation.](image-url)
variety of different signaling and structural proteins in all classes of eukaryotic species. Different computational methods were used to predict the localization and structure of SO. Transmembrane domains or a signal peptide were not detected in SO. Localization prediction using PSORTII gave a 60.9% likelihood for nuclear and 30.4% for cytoplasmic localization of SO.

BLAST searches of the NCBI database with the predicted so ORF (NCU02794.1) revealed orthologs in the genomes of a number of filamentous ascomycete species. The closest ortholog is a gene encoding a protein of unknown function in *Sordaria macrospora* (locus CAE83713; 76% aa identity). The genome of *Magnaporthe grisea* also contains an ortholog of so (MG01636.4; 58% aa identity). Similarly, a gene coding for a protein with 57% aa identity to SO (AN5776.2) was identified in the genome of the more distantly related filamentous ascomycete species, *Aspergillus nidulans*. In contrast, searches of the genomes of *S. cerevisiae*, *Candida albicans*, *Schizosaccharomyces pombe*, or the basidiomycete species, *Ustilago maydis* and *Cryptococcus neoformans*, did not reveal so orthologs.

The C-terminal part of the so protein (aa 781 to 1237) shows similarity to a hypothetical protein of *N. crassa* (NCU03220.1). Homologs of NCU03220.1 were identified in the genomes of other filamentous ascomycete species, such as *M. grisea* (MG03714.4) and *A. nidulans* (AN3582.2), as well as in *S. cerevisiae* (Ygr266), *C. albicans* (SC5314) and basidiomycete species, *U. maydis* (UM04813.1) and *C. neoformans* (CNBE0710), but not in the genome of *S. pombe*. Homologs to NCU03220.1 were not found in other eukaryotic species.

**DISCUSSION**

In this study, we phenotypically characterized so, a hyphal fusion mutant in *N. crassa*. We showed that so is allelic with *ham-1*, cloned the so gene, identified the mutation in the original so mutant and confirmed our results by generating RIP mutants of the so gene. Orthologs of the so gene were identified only in the genomes of euascomycete species. Although fusion processes occur in other ascomycete species during mating, such as in *S. cerevisiae* (9) and during dikaryon formation and vegetative growth in basidiomycete species (11, 25), the requirement for so during anastomosis is apparently restricted to filamentous ascomycete species.

To characterize the fusion defect of so, we assessed its cell fusion phenotype during different stages of development. Although germling fusions between so and wild-type strains were never observed by microscopy, it is apparent that the so mutant can form heterokaryons with a wild-type strain by forcing auxotrophic markers. However, the frequency of formation of such (so + WT) heterokaryons is reduced (~300-fold). In contrast, heterokaryon formation between another hyphal fusion (*ham-2*) mutant and a wild-type strain is reduced more than ~1,000-fold (62). While the so mutant exhibits a defect in germling and hyphal fusion during vegetative growth, the formation of CATs was observed in so mutants, albeit at a lower frequency than that in wild-type conidia. These results are in contrast to the other fusion mutants in *N. crassa*, *ham-2*, *nrc-1*, and *mak-2* strains, where CAT formation was never observed during conidial germination (47). These observations suggest that SO functions downstream of the initiation of germling fusion via activation of the *mak-2* MAPK pathway and the putative transmembrane protein, HAM-2 (39, 62). However, although so mutants formed CATs, so germlings did not home toward other conidia nor did they attract wild-type germlings.

These observations suggest that the so mutant is defective in the biochemical machinery involved in the synthesis and/or secretion of the extracellular chemoattractant and the signaling apparatus involved in its perception and/or transduction which results in homing. It is presently not clear what the relationship is between CAT formation by so mutants during germling fusion and the inability of so mutants to undergo hyphal fusion within a colony.

Although so mutants were deficient in germling and hyphal fusion, cell fusion processes associated with sexual development were not affected. Trichogynes of so perithecia showed directed growth to conidia of the opposite mating type, and trichogyno-conidium fusion occurred. In addition, fusion of crozier cells in so homoyzogous crosses was not affected. Developmental features associated with postfertilization events, such as protoperithecial enlargement, melanization, and ostiole production, were not observed when so was used as a female in crosses. The female fertility defect of so could be complemented by heterokaryon formation with the *a"m1"* strain. This complementation is in contrast to formation of dikaryotic ascogenous hyphae, where complementation of sexual defects by an *a"m1"* helper strain does not occur (40, 44). These data indicate that SO has a role in developmental processes after trichogyno-conidium fusion, but before the development of ascogenous hyphae and the dikaryotic crozier. Events following trichogyno-conidium fusion until the development of fertile ascogenous hyphae (where the dikaryon becomes established) have not been well characterized in any filamentous ascomycete species. Dikaryotic hyphal formation in basidiomycete species, which is the predominant growth form, has been genetically characterized and shown to be regulated by mating-type loci (11, 25). However, dikaryon formation in basidiomycete species requires fusion between monokaryotic hyphae or yeast cells, rather than fertilization between specialized reproductive structures, as in *N. crassa*. It is not clear where so might be required during this little understood phase of sexual development in filamentous ascomycete species. The other hyphal fusion mutants in *N. crassa*, *mak-2*, *nrc-1*, and *ham-2* strains, fail to make protoperithecia, and so their role during trichogyno-conidium fusion cannot be evaluated (39, 62). As with so, a cross between [(*a"m1"* + *mak-2*) × *mak-2*] forms asci and ascospores (39), and although [(*a"m1"* + *ham-2*) × *ham-2*] crosses are lethal (62), fusion of crozier cells was still observed (N. B. Raju, D. Rasmussen, and N. L. Glass, unpublished observations).

Although cell fusion processes associated with vegetative growth versus sexual development serve different developmental functions, they both require extracellular communication and chemotropic interactions, followed by cell wall breakdown, membrane merger, and pore formation (14, 15, 20). The fact that so affects vegetative germling and hyphal fusion, but not the attraction or fusion of trichogynes to conidia of the opposite mating type indicates that at least chemotropic interactions in these two processes are different and independent, a finding supported by other studies. For example, trichogynes of *N. crassa* strains that carry a mutation in the putative mating
pheromone receptor, pre-1, fail to show chemotropic behavior toward a male-mating partner and are therefore female sterile (27). However, vegetative cell fusion in pre-1 mutants was unaffected. Furthermore, both mat a and mat A mating-type mutants of N. crassa are competent to undergo hyphal fusion, even though such mutants are sterile (16). Altogether, these data suggest that different cell fusion regulatory pathways may be involved in vegetative fusion versus trichogyne-conidium and crozier fusion during sexual development.

Although the morphology of hyphal and germling fusions in filamentous ascomycete species has been subject to a number of studies (4, 20, 28, 29, 47, 48), the function of the interconnected hyphal colony during fungal developmental processes or growth is only poorly understood. so and the other known fusion mutants of N. crassa exhibit pleiotropic phenotypes (30, 39, 62). Anastomosis within a colony presumably contributes to distribution of nutrients throughout a colony (46). The inability to partition metabolites could affect growth phenotype or growth rates and could possibly explain the fact that the colony margins of the so mutants are irregular as compared to those of a wild-type strain. In addition to ensuring nutrient distribution, anastomosis may also facilitate signaling associated with developmental processes (14). The altered growth and conidiation pattern observed in so mutants may occur as a direct result of the inability to undergo anastomosis. Understanding the role of so and its relationship to other components of the fusion machinery will help us to learn more about the mechanism and function of hyphal fusion in filamentous fungi and provide an understanding on the more general question of eukaryotic cell fusion.

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