Pheromones Are Essential for Male Fertility and Sufficient To Direct Chemotropic Polarized Growth of Trichogynes during Mating in Neurospora crassa

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Neurospora crassa is a self-sterile filamentous fungus with two mating types, mat A and mat a. Its mating involves chemotropic polarized growth of female-specific hyphae (trichogynes) toward male cells of the opposite mating type in a process involving pheromones and receptors. mat A cells express the ccg-4 pheromone and the pre-1 receptor, while mat a strains produce mRNA for the pheromone mfa-1 and the pre-2 receptor; MFA-1 and CCG-4 are the predicted ligands for PRE-1 and PRE-2, respectively. In this study, we generated Δccg-4 and Δmfa-1 mutants and engineered a mat a strain to coexpress ccg-4 and its receptor, pre-2. As males, Δccg-4 mat A and Δmfa-1 mat a mutants were unable to attract mat a and mat A trichogynes, respectively, and consequently failed to initiate fruiting body (perithelial) development or produce meiotic spores (ascospores). In contrast, Δccg-4 mat a and Δmfa-1 mat A mutants exhibited normal chemotropic attraction and male fertility. Δccg-4 Δmfa-1 double mutants displayed defective chemotropism and male sterility in both mating types. Heterologous expression of ccg-4 enabled mat a males to attract mat a trichogynes, although subsequent perithelial differentiation did not occur. Expression of ccg-4 and pre-2 in the same strain triggered self-stimulation, resulting in formation of barren perithecia with no ascospores. Our results indicate that CCG-4 and MFA-1 are required for mating-type-specific male fertility and that pheromones (and receptors) are initial determinants for sexual identity during mate recognition. Furthermore, a self-attraction signal can be transmitted within a strain that expresses a pheromone and its cognate receptor.

In heterothallic (self-sterile) fungi, pheromones play an important role in mating by facilitating recognition between strains of opposite mating types (5, 8). Pheromones are secreted from cells as small diffusible peptides that can attract mates of the opposite mating type without cell-cell contact. Binding of pheromones to receptors on the surface of sexually compatible cells launches a signal transduction pathway, often involving a mitogen-activated protein kinase cascade. Cells then undergo morphological and physiological changes prior to fusion. Pheromone precursor genes in the yeast Saccharomyces cerevisiae have been extensively studied (24). The mating factor α gene (MFα) encodes a precursor containing multiple repeats of the pheromone sequence bordered by Kex2 protease processing sites. The mating factor α gene (MFα) encodes a short peptide with a C-terminal CAAX motif (C, cysteine; A, aliphatic; X, any amino acid residue). The mature α-factor is highly hydrophobic due to prenylation at the cysteine residue, while the mature α-factor is hydrophilic and unmodified. These two classes of pheromone precursor genes have also been identified in several heterothallic filamentous ascomycetes, including Cryptococcina parasitica (50), Magnaporthe grisea (39), Podospora anserina (11), and Neurospora crassa (4, 21), while only the MFα-related class has been found in basidiomycete fungi (5, 31, 44).

Neurospora crassa is a heterothallic filamentous fungus with two mating types, mat A and mat a. It undergoes a morphologically more complex process of sexual reproduction than unicellular yeasts (35). Under nitrogen starvation, light, and low temperature, vegetative hyphae differentiate into a coiled hyphal mass (ascogonium) and form a multicellular female reproductive structure (protoperithecium), from which specialized receptive hyphae (trichogynes) are extended. The trichogynes grow toward and fuse exclusively with cells of the opposite mating type (males). The directed growth and fusion are mediated by chemotropic communication between pheromones diffused from male cells and their cognate receptors on trichogynes (3, 20). Following fusion, a male nucleus migrates into the protoperithecium and this fertilized protoperithecium develops into a fruiting body (perithecium). Within the ascogonium, nuclei of opposite mating types undergo several synchronous mitotic divisions and migrate in pairs to the developing asci (croziers), where they undergo karyogamy and subsequent meiosis (35, 36). Normal development of a perithecium includes initial rapid enlargement and melanization, formation of an ostiole and a beak at the tip, and generation of the final meiotic products, the haploid ascospores.

In N. crassa, both classes of pheromone precursor genes, ccg-4 and mfa-1, have been identified (4, 21). These genes encode precursor polypeptides structurally similar to the α-factor and a-factor of S. cerevisiae, and their expression is directly regulated by the transcription factors encoded by the mating-type genes mat A and mat a, respectively. Null ccg-4 mutants have not been reported, while mfa-1 gene mutants were analyzed in a recent study (21). The mutations in the mfa-1 gene were generated by the repeat-induced point (RIP) mutation approach (6, 38) and contained point mutations in both the open reading frame (ORF) and 3′ untranslated region (UTR) (21). The mfa-1RIP null strains displayed male sterility in the

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mat a mating type due to the inability to attract mat A trichogynes. They also possessed additional defects in female sexual development, ascospore production, and vegetative growth. These pleiotropic phenotypes suggested that the pheromone precursor genes may have functions beyond their role in fertilization. Recent studies of several fungi have suggested that cultures growing on solid medium, μL of a conidial suspension was inoculated onto the center of VM or SCM plates. VM plate cultures were grown in the dark at 30°C for 3 days, while SCM plate cultures were grown for 6 days at 25°C under constant light. For RNA extractions, cultures were grown on plates overlaid with cellophane (Bio-Rad Laboratories, Hercules, CA), and differentiated tissues were scraped from the plates and frozen immediately in liquid nitrogen until use. Plasmids were maintained in Escherichia coli DH5α (17).

ccg-4 and mfa-1 gene replacement constructs. The ccg-4 and mfa-1 gene replacement constructs pHK71 and pHK72, respectively, were made by an efficient knockout procedure for S. cerevisiae. For each gene, 5′ and 3′ gene-flanking fragments and a selectable marker cassette were prepared by PCR. These fragments were then cotransformed with a yeast shuttle vector into S. cerevisiae.

In this study, we created strains with complete deletions of the ccg-4 and mfa-1 ORFs. We characterized single and double mutants for phenotypes during sexual and asexual growth and development. We also analyzed a strain engineered to coexpress a compatible receptor-pheromone pair. Our results indicate that the ccg-4 and mfa-1 coding sequences are required for male fertility in a mating-type-specific manner and that heterologous expression of a pheromone gene in male cells can direct chemotropic polarized growth of trichogynes from an otherwise incompatible mating-type background.

### MATERIALS AND METHODS

**Strains, media, and culture conditions.** The *N. crassa* strains used in this study are listed in Table 1. The strains were grown on Vogel’s minimal medium (VM) (46) for vegetative growth and on synthetic crossing medium (SCM) (47) to induce development of protoperithecia. Sorbose-containing medium was used to facilitate colony formation on plates (12). Where indicated, hygromycin B was used at 200 μg/ml in media. 1-Histidine (100 μg/ml), pantothetic acid (10 μg/ml), adenine (10 μg/ml), and nicotinic acid (10 μg/ml) were supplemented for auxotrophic strains. Seven-day-old conidia were used to inoculate all cultures. For cultures growing on solid medium, μL of a conidial suspension was inoculated onto the center of VM or SCM plates. VM plate cultures were grown in the dark at 30°C for 3 days, while SCM plate cultures were grown for 6 days at 25°C under constant light. For RNA extractions, cultures were grown on plates overlaid with cellophane (Bio-Rad Laboratories, Hercules, CA), and differentiated tissues were scraped from the plates and frozen immediately in liquid nitrogen until use. Plasmids were maintained in *Escherichia coli* DH5α (17).

### TABLE 1. *N. crassa* strains

| Straina | Relevant genotype | Comments | Source or reference |
|---------|------------------|----------|---------------------|
| 74A; FGSC 2489 | Wild type, *mat A* | FGSC |
| ORSα; FGSC 4200 | Wild type, *mat A* | FGSC |
| 74a; FGSC 988 | Wild type, *mat A* | FGSC |
| 90-2; FGSC 9718 | Recipient for transformation | FGSC |
| A<sup>m<sup>aa</sup></sup>; FGSC 4570 | *mat A* sterile mutant | FGSC |
| a<sup>aa</sup>; FGSC 4564 | *mat A* sterile mutant | FGSC |
| fl A; FGSC 4317 | | FGSC |
| fl a; FGSC 4318 | | FGSC |
| his-3 A; FGSC 6103 | | FGSC |
| his3a | his-3 mat A | 23 |
| pan-2 A | pan-2 mat A | Progeny of pan-2 A × 74a |
| pan-2 a | pan-2 mat a | Progeny of pan-2 A × 74a |
| m1 | Δccg-4::hph mat a heterokaryon | This study |
| m5 | Δmfa-1::hph mat a heterokaryon | This study |
| c(1)A | Δccg-4::hph mat A | This study |
| m(1)A | Δmfa-1::hph mat A | This study |
| m5A) | Δmfa-1::hph mat A | This study |
| c(1)A his-3 | Δccg-4::hph his-3 mat A | This study |
| c(1)A his-3 | Δccg-4::hph his-3 mat a | This study |
| c(1)A pan-2 | Δccg-4::hph pan-2, mat A | This study |
| c(1)A pan-2 | Δccg-4::hph pan-2, mat a | This study |
| m(1)A his-3 | Δmfa-1::hph his-3 mat A | This study |
| m(1)A his-3 | Δmfa-1::hph his-3 mat a | This study |
| m(1)A pan-2 | Δmfa-1::hph pan-2 mat A | This study |
| m(1)A pan-2 | Δmfa-1::hph pan-2 mat a | This study |
| c4m1A | Δccg-4::hph Δmfa-1::hph mat A | This study |
| c4m1a | Δccg-4::hph Δmfa-1::hph mat a | This study |
| c4A70 | his-3::Pccg-4::ccg-4 Δccg-4::hph mat A | This study |
| m1A70 | his-3::Pccg-4::ccg-4 Δmfa-1::hph mat a | This study |

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In the **ccg-4** gene replacement construct, pHK71 (Fig. 1A), the 1,080-bp **ccg-4** region (spanning from 75 bp upstream to 105 bp downstream of the 900-bp ORF) was replaced with the 1.4-kb **hph** marker cassette. pHK71 also contains 1.3 kb of 5′ and 1.3 kb of 3′ flanking DNA. In the **mfa-1** gene replacement construct, pHK72 (Fig. 1B), the 380-bp **mfa-1** region (extending from 218 bp upstream to 87 bp downstream of the 75-bp ORF) was replaced with the 1.2-kb **hph** cassette. pHK72 also contains 1.2 kb of 5′ and 1.2 kb of 3′ flanking DNA.

**Isolation of Δ**ccg-4-, Δ**mfa-1**, and Δccg-4Δmfa-1 mutants.** The 4-kb and 3.8-kb knockout cassettes for **ccg-4** and **mfa-1** were amplified from pHK71 and pHK72, respectively, by using the 5′ and 3′ primer pair for each gene (Fig. 1). The PCR fragments were purified and electroporated into *N. crassa Δmss-5Δbar-** mutant strain 90-2 (resistant to phosphinothricin [10]), as previously described (18, 45). The **Δmss-5** strain was used because of its high frequency of homologous integration of transforming DNA (10, 29). Heterokaryotic transformants with pHK71 were initially isolated based on their resistance to hygromycin and then examined by Southern analysis in order to identify **Δccg-4::hph** or **Δmfa-1::hph** gene replacement strains. *N. crassa* genomic DNA was isolated from transformants with the Puregene DNA kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instructions. Genomic DNAs for **Δccg-4::hph** and **Δmfa-1::hph** were digested with EcoRI, and blots were probed with the 4-kb knockout cassette; those for **Δmfa-1::hph** were digested with Xhol, and blots were probed with the 3.8-kb knockout cassette. The identified heterokaryons were subsequently crossed to wild-type strain 74A in order to obtain homokaryotic **Δccg-4::hph** or **Δmfa-1::hph** strains without the **Δmss-5** mutation in the genetic background. **Δmss-5** strains were identified by virtue of their sensitivity to phosphonothricin (32). Homokaryotic progeny were selected after being plated on sorbose medium containing hygromycin and verified by Southern analysis. The homokaryons were crossed to *his-3* or *pan-2* strains of the opposite mating type in order to obtain auxotrophic **Δccg-4::hph** or **Δmfa-1::hph** strains for analysis of heterokaryotic formation. In order to isolate **Δccg-4Δmfa-1** double mutants, **Δccg-4** and **Δmfa-1** strains were crossed to each other. These progeny were selected on hygromycin-containing sorbose medium, and **ccg-4** and **mfa-1** genotypes were verified by Southern analysis.

**Plasmid construction and strain generation for heterologous expression of ccg-4.** During this study, a new *his-3* targeting vector for *N. crassa*, phHK40, was constructed. phHK40 is a pRS416 derivative carrying the *his-3* gene and its 3′ flank on a 6-kb fragment. The 6-kb fragment was amplified from genomic clone G21G10 as two 3-kb fragments (the left and the right flank) with primer pair 1 and 2 and primer 1 and 4 in PCRs, respectively (Table 2). The two fragments were then sequentially inserted into pRS416. The left flank was ligated into pRS416 with the HindIII and EcoRI sites, and the right flank was then ligated into pRS416 with the Xhol and NotI sites. To facilitate the cloning process, EcoRI, Xhol, and NotI sites were generated into primers 2, 3, and 4, respectively (Table 2), and the original EcoRI site in the genomic region was removed by altering the sequence in primer 4 (Table 2). Approximately 20 nucleotides of genome sequence between the two flanks were deleted during this process.

Plasmid pHK70 is a pHK40 derivative carrying the *ccg-4* ORF under the control of the 0.9-kb **ccg-4** promoter region (16). The **ccg-4** ORF was amplified from the XAS cosmid with primers 5 and 6 (Table 2), while the **ccg-4** promoter fragment and **gfp** gene were isolated from the plasmid pMF272 as a NotI-EcoRI restriction fragment (16). The NotI- and EcoRI-digested Pccg-4-gfp fragment was ligated into XbaI- and EcoRI-digested pHK40, yielding pHK42. The **ccg-4** ORF was then inserted into XbaI- and EcoRI-digested pHK42 as a replacement of *gfp*, yielding pHK70. XbaI and EcoRI sites were generated into primers 5 and 6, respectively, in order to facilitate the cloning process.

To examine the effect of heterologous expression of **ccg-4** and to ensure that **ccg-4** was the only pheromone produced, pHK70 was electroporated into the **Δmfa-1**-his-3 strain m(1)a. The Δccg-4-his-3 strain c(1)A was also transformed with pHK70 to verify that **ccg-4** could be expressed from the his-3 locus. Heterokaryotic transformants were selected on minimal medium and then checked for the proper integration event by Southern analysis using the 8.8-kb HindIII fragment from pRAUW122 (which contains the his-3 gene [1]) as a probe (data not shown). All genomic DNAs were digested with HindIII. Homokaryons con-
taining nuclei with a single copy of the vector targeted to the his-3 locus were purified by the microconidiation procedure (14) and verified by Southern analysis (data not shown).

**Northern analysis.** Total RNA was isolated using the PURESCRIPT RNA isolation kit (Genta Systems), according to the manufacturer’s instructions. Northern analysis was performed as previously described (42) with 10 or 30 µg of total RNA isolated from the tissues indicated in the figure legends. The entire ORF of ccg-4 was amplified from the X5A6 cosmid with primers 7 and 8 and used as a probe after purification. For the mfa-1 probe, a 278-bp fragment encompassing the ORF was amplified from the G17B9 cosmid with primers 9 and 10 (Table 2).

**Phenotypic analysis.** (i) Growth rate, conidiation, heterokaryon formation, and CAT fusion assays. Apical extension rates were examined on VM and SCM plates by measuring colony diameters, as described previously (18). Morphology in submerged cultures was examined by using liquid VM inoculated with conidia to a final cell density of 10^6 conidia/ml, and cultures were grown for 16 h at 30°C with agitation (200 rpm) (19). The ability to form heterokaryons was tested by observing colony formation between two strains with different auxotrophic markers (his-3 or pan-2 [12]). Conidial anastomosis tube (CAT) (37) fusion assays were performed as described by Fleißner et al. (15): 3 × 10^6 conidia were germinated on VM plates at 25°C and analyzed for germinating fusion at 3 to 5 h after inoculation. Microscopic observation was made with a BX41 fluorescent microscope with UM Plan Fluorite objective lenses (Olympus America, Lake Success, NY).

(ii) **Fertility tests.** To test female fertility, strains were grown on SCM and fertilized with wild-type conidia of the opposite mating type. For male fertility, dilute conidial suspensions of strains were applied directly to 7-day-old SCM cultures of fl mat a and fl mat A strains containing fully differentiated protoperithecia. Mating types of mutants were also determined by such crosses. For male-sterile strains, mating types were determined by growing the mutants on SCM and fertilizing them with wild-type conidia of each mating type. The Δccg-4, Δmfa-1, and Δccg-4 Δmfa-1 strains were also crossed to sibling mutant strains in order to detect possible recessive traits affecting sexual development. The formation of protoperithecia and development of perithecia were examined microscopically by using a SZX9 stereomicroscope with an ACH 1 × objective lens, and images were photographed with a C-4000 digital camera (Olympus America).

(iii) **Trichogyne assay.** Chemotrophic attraction between trichogynes and conidia of opposite mating types was examined as described previously (3, 20). When Δccg-4, Δmfa-1, or Δccg-4 Δmfa-1 strains were assayed as a male, an opposite mating-type fl strain was used as a female due to its high fertility and inability to produce macroconidia (25). Orientation and growth of trichogynes were monitored and photographed by using a BX41 fluorescence microscope with UM Plan Fluorite objective lenses and a PM-C3SB camera (Olympus America).

**RESULTS**

ccg-4 and mfa-1 are required for male fertility in mat A and mat a strains, respectively. In order to assay biological functions of ccg-4 in *N. crassa*, its entire ORF was deleted by targeted gene replacement with a hygromycin B resistance gene cassette (*hph*) (Fig. 1A). Genomic DNA from hygromycin-resistant transformants was subjected to Southern analysis. DNAs from Δccg-4 strains were digested with EcoRI, and blots were probed with the 4-kb fragment from pHK71. The heterokaryotic strains with Δccg-4 nuclei were identified by 3.0- and 1.1-kb hybridizing fragments in addition to a 3.7-kb hybridizing fragment representing wild-type nuclei (data not shown). Homokaryotic Δccg-4 strains were obtained by crossing heterokaryotic primary transformants to the wild type, with selection for growth of progeny on hygromycin. Replacement of the gene in all nuclei was confirmed by Southern analysis (Fig. 1A). Northern analysis was used to measure ccg-4 transcript levels in RNA isolated from macroconidia (Fig. 2). Consistent with previous results (4), ccg-4 is expressed in mat A, but not mat a, wild-type *N. crassa* strains. ccg-4 could not be detected in *mat A* or *mat a* Δccg-4 mutants, further verifying the Δccg-4 mutation (Fig. 2).

**FIG. 2.** Verification of Δccg-4 and Δmfa-1 strains by Northern analysis. Strains are *WT* and *OR* strains (*WT*, c11A and c11a (Δccg-4), and m11A and m11a (Δmfa-1)). Total RNA was isolated from conidia of the indicated strains, and approximately 10 µg was subjected to Northern analysis using the ccg-1 or mfa-1 ORF as a probe (see Materials and Methods).

The ccg-4 gene is predicted to encode a peptide pheromone. Thus, we first examined Δccg-4 strains for defects in male fertility. For these assays, we used fl mat A or fl mat a strains as the female parents, due to their high fertility and inability to produce macroconidia (12). Consistent with previous results, conidia from wild-type *mat A* and *mat a* strains were able to attract trichogynes and fertilize protoperithecia from fl females of the opposite mating type (Fig. 3). Similarly, conidia from Δccg-4 mat a strains were able to fertilize fl mat A protoperithecia and to trigger normal chemotrophic attraction from fl mat A trichogynes (Fig. 3). In contrast, Δccg-4 mat A strains were completely unable to initiate perithecial development when crossed as males to fl females (Fig. 3A). The protoperithecia did not show any signs of fertilization, such as enlargement or melanization after application of male cells. When examined microscopically, it was noted that conidia of Δccg-4 mat A strains did not direct the growth of or fuse with fl mat a trichogynes (Fig. 3B). These results are consistent with the proposed function of ccg-4 as a pheromone specific to *mat A* strains of *N. crassa*.

The previously reported RIP-induced *mfa-1* disruption mutation included numerous point mutations in the noncoding region (21). The pleiotropic phenotypes noted for the *mfa-1* RIP strains suggested multiple functions for *mfa-1* during the *N. crassa* life cycle, as well as a potential role for its long 3′ UTR. In order to explore these possibilities, we generated Δmfa-1 strains in which only the *mfa-1* ORF was replaced with *hph* (Fig. 1B). Transformants were selected based on their resistance to hygromycin. Genomic DNA from candidate transformants was subjected to Southern analysis following digestion with XhoI, with the 3.8-kb fragment from pHK72 as the probe. Δmfa-1 nuclei contain a 3.6-kb hybridizing fragment, while that from wild-type nuclei is 2.7 kb. Homokaryotic Δmfa-1 strains were isolated from crosses of heterokaryons to the wild type, and the presence of the gene replacement in all nuclei was verified by Southern analysis (Fig. 1B). The Δmfa-1 mutation was further validated by the absence of the *mfa-1* transcript in conidia from Δmfa-1 mutants of either mating type (Fig. 2). In keeping with previous observations (4, 21), wild-type *mat a* (but not *mat A*) strains express *mfa-1*. Δccg-4 Δmfa-1 double mutants were created by using sexual crosses between single mutants (Table 2).

We analyzed the male fertility of Δmfa-1 and Δccg-4 Δmfa-1 strains. Δmfa-1 mat A strains displayed normal male fertility and chemotrophic attraction to fl females of the opposite mating
type (Fig. 3). In contrast, conidia from /H9004 mfa-1 mat a strains did not trigger perithecial development when crossed as males to fl mat A females (Fig. 3A). This defect stems from the inability of /H9004 mfa-1 mat a mutants to direct the growth of and fuse with fl mat A trichogynes (Fig. 3B).

/H9004 ccg-4 /H9004 mfa-1 double mutants displayed male sterility in both mating types (Fig. 3A). Perithecial development was completely absent in crosses in which the double mutants were used as males (Fig. 3A), and these strains were unable to attract and fuse with trichogynes of females of the opposite mating type (Fig. 3B).

The coding sequences for ccg-4 and mfa-1 are not required for female fertility. Previous studies demonstrated that the ccg-4 and mfa-1 genes are abundantly expressed in unfertilized and fertilized sexual tissues (4, 21, 28). We followed up on these observations by analyzing transcript levels for all pheromone receptors and pheromone genes in /H9004 mfa-1 and /H9004 ccg-4 single and double mutants by using RNA isolated from SCM plate cultures (Fig. 4A). Levels of the ccg-4 and mfa-1 transcripts are affected by no more than twofold in /H9004 and /H9004 ccg-4 mutants, respectively. Similarly, levels of pre-2 were relatively unchanged in the Δccg-4 and Δmfa-1 mutants in comparison to the wild type (Fig. 4A). However, we observed a significant and reproducible reduction in the amount of pre-1 transcript in Δccg-4 (mat A) mutants (Fig. 4A). This result is surprising, as CCG-4 is the ligand for PRE-2, not PRE-1, and would not be predicted to have a receptor target in mat A cells.

We extended our analysis of possible roles for pheromone genes in female sexual development by examining /H9004 ccg-4 and /H9004 mfa-1 mutants for defects in aspects of female fertility. Both mating types of /H9004 ccg-4 strains displayed normal differentiation of protoperithecia (Fig. 4B) and trichogynes (data not shown) on SCM. The protoperithecia developed into perithecia and produced ascospores when crossed with wild-type males (Fig. 4B). In contrast to results previously reported for RIP-induced mutants, the /H9004 mfa-1::hph strains formed fully differentiated protoperithecia with trichogynes on SCM and underwent normal sexual development and produced viable ascospores as females (Fig. 4B). Δccg-4 Δmfa-1 double mutants also formed...
normal protoperithecia and underwent normal perithecial development with wild-type conidia of the opposite mating type (Fig. 4B). Trichogynes of \(H9004\) ccg-4, \(H9004\) mfa-1, and \(H9004\) ccg-4/mfa-1 strains of both mating types displayed normal chemotropism and fusion with wild-type conidia of the opposite mating type (data not shown).

The ccg-4 and mfa-1 ORFs have no obvious roles during vegetative growth or development. Deletion of the ccg-4 or mfa-1 ORF singly or in combination did not affect aspects of vegetative growth or development in either mating type. Hyphal apical extension was similar to that of the wild type on solid VM or SCM medium (data not shown). Differentiation of aerial hyphae and macroconidiation were normal (data not shown). The morphology of the pheromone mutants in submerged cultures was similar to that of wild-type strains (data not shown).

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mat \(a\) conidia that express ccg-4 can attract mat \(a\) trichogynes during mating. The ccg-4 transcript is normally produced only in the mat \(A\) background, as expression of pheromone precursor genes is modulated by the transcription factors encoded by the mating-type genes in \(N.\ crassa\) (Fig. 2 and 4A) (4). The CCG-4 peptide is hypothesized to interact with the PRE-2 protein, the mat \(a\) pheromone receptor, for mate recognition and sexual development in \(N.\ crassa\) (4, 20).

When placed under control of the ccg-1 promoter (2, 26), the ccg-4 gene was abundantly expressed in conidia of the m1a70 (mat \(a\)) and c4A70 (mat \(A\)) strains (Fig. 6A). The ectopically expressed ccg-4 was functional, as it fully reinstated male fertility to \(\Delta ccg-4\) mat \(A\) mutants (Fig. 6B). Similar to mat \(A\) wild-type strains, conidia from the c4A70 strain were able to attract and fuse with fl mat \(a\) trichogynes and go on to complete perithecial
development and produce viable ascospores (Fig. 6B). Furthermore, conidia from the m1a70 strain were able to trigger fl mat a protoperithecia to enlarge and darken and to attract fl mat a trichogynes despite the mating-type incompatibility between the two gametes (Fig. 6B). Interestingly, individual fl mat a trichogynes would coil once or twice around an m1a70 conidium and then resume growth in the direction of another conidium. The trichogyne would subsequently coil in a similar fashion around the new conidium; this behavior of the trichogyne would repeat several times. In spite of participating in multiple coiling events with several m1a70 conidia, no fully developed perithecia were ever produced in the fl mat a protoperithecia (Fig. 6B). The enlarged and darkened bodies neither formed detailed exterior structures, such as beaks or ostioles (Fig. 6B), nor contained interior features, such as ascogenous hyphae (data not shown).

Coexpression of CCG-4 and PRE-2 in mat a strains does not induce self-fertility. The filamentous fungus *Sordaria macrospora* is closely related to *N. crassa* yet does not require a mating partner in order to complete the sexual cycle. The homothallic *S. macrospora* coexpresses two pheromone precursor and two pheromone receptor genes (33, 34), a compatible pair of which were demonstrated to be functional in a recent study (27). In light of these results, we examined the *N. crassa* m1a70 strains

FIG. 5. Germling fusion assay. Formation of CATs and/or fusion between germinating conidia (indicated by arrows) was assessed microscopically. Strains are the same as in Fig. 3.
FIG. 6. Fertility and sexual development of strains with heterologous expression of ccg-4. Strains are 74A (WT [wild type] mat A), ORSa (WT mat a), FGSC 4318 (fl mat a), c4A70 (Δccg-4 mat A, ccg-4ec), and m1a70 (Δmfa-1, mat a, ccg-4ec). (A) Effects on gene expression. Total RNA was isolated from SCM plate cultures, and 10 μg or 30 μg was used to prepare blots for hybridization with the ccg-4 and pre-2 ORF probes, respectively. (B) Male fertility. Direct application of conidia from wild-type, c4A70, or m1a70 strains to fl mat a protoperithecia (top panels) and chemotropic attraction of fl mat a trichogynes toward the same conidia (bottom panels) are shown. For the top panels, arrows indicate perithecia. In the bottom panels, arrows show the direction of trichogyne growth while asterisks indicate coiling events. The bottom left-most arrow for the fl mat a × m1a70 interaction indicates a trichogyne that is emerging from below the surface of the agar. (C) Sexual development of m1a70, a strain coexpressing ccg-4 and pre-2, a compatible pair of pheromone and pheromone receptor genes. A wild-type (ORSa) × wild-type (74A) cross (left) and an unfertilized wild-type (ORSa) SCM culture (middle) are shown as controls for normal perithecial and protoperithecial development, respectively. Apparent perithecial development in the m1a70 strain in the absence of a mating partner is presented in the right-hand frame. White arrows indicate enlarged, darkened protoperithecia, while black arrows show normal-sized unfertilized protoperithecia. The inner box in the right-hand frame shows a large, melanized fruiting body that represents the fate of approximately 5% of the protoperithecia formed in this strain background.
for self-fertility traits, since they coexpress ccg-4 and pre-2, a compatible pair of pheromone and pheromone receptor genes (Fig. 6A).

When subjected to nitrogen starvation (SCM plates), the mfa-1 strains initially displayed normal protoperithecial formation. However, with continued incubation (and in the absence of male cells of the opposite mating type), the protoperithecia grew darker and larger than those of a wild-type strain cultured alone for the same length of time (Fig. 6C). Approximately 5% of the bodies became enlarged to the size of perithecia observed in wild-type crosses (Fig. 6C, inset). However, the structures lacked defining features of perithecia, such as beaks and ostioles, and ascospores were never produced (data not shown). These results are consistent with a block in sexual development prior to the onset of meiosis and ascospore formation.

DISCUSSION

In heterothallic filamentous fungi, the sexual identity of a strain is established by the mating-type locus (22). Pheromones function as initial determinants during mate recognition, since expression of their precursor genes is normally restricted to specific mating types (4, 11, 39, 50). Recently, biological assays have been performed with deletion mutants of Mf1-1 in C. parasitica and of mfp and mfm in P. anserina (11, 43), thus demonstrating that pheromones are essential for male fertility in heterothallic filamentous fungi. In this study, through microscopic observations of the mating response and perithecial development, we demonstrate a requirement for pheromones in male cells for chemotropic recognition and plasmogamy with cells of the opposite mating type during mating in N. crassa. CCG-4 and MFA-1 are necessary for conidia (and presumably other cell types that can function as males) in mat A and mat a backgrounds, respectively, to direct the polarized growth of and to fuse with trichogynes of the opposite mating type. In contrast to their roles in male fertility, pheromones are dispensable for female functions during sexual reproduction.

We have previously shown that both chemotropic behavior and perithecial development require that females contain the appropriate pheromone receptor (20), the heterotrimeric G protein subunit GNA-1 (18, 20), and both subunits of the Gβγ dimer in N. crassa (23, 49). These four proteins do not have apparent roles in male fertility. Thus, sexual reproduction in N. crassa requires that females contain this G-protein-coupled receptor (GPCR)/G protein signal transduction pathway, while males need only produce the GPCR ligand.

The inability of conidia from Δccg-4 mat A and Δmfa-1 mat a strains to fuse with fl mat a and fl mat A trichogynes even when in direct contact contrasts with their ability to fuse with neighboring conidia and vegetative hyphae, as observed during vegetative CAT fusion (37) and forced heterokaryon formation (12) assays. These results indicate that pheromones are required not for vegetative cell fusion but for fusion of conidia with specialized female reproductive cells. In contrast, the N. crassa so (soft) mutant displays defects in cell-cell fusion that are quite different from those of Δccg-4 mat A and Δmfa-1 mat a strains. The so gene encodes a protein that is predicted to be involved in protein-protein interactions (15). Conidia of so mutants exhibit defects in fusion with other vegetative cells (conidia or hyphae) but are able to fuse with trichogynes of females of the opposite mating type, displaying normal male fertility (15). These data suggest that vegetative cell fusion and sexual cell fusion are regulated by different mechanisms of cell-cell communication in N. crassa.

Deletion mutants lacking the ccg-4 or mfa-1 ORF did not display obvious defects in vegetative development and female fertility. The lack of a defect in vegetative or protoperithecial development for N. crassa pheromone mutants is perhaps surprising, as high levels of pheromone expression are observed during conjugation and protoperithecial development (references 4, 21, and 28 and this study). The data for Δmfa-1 mutants contrasts with the results previously reported for RIP-induced mutants, suggesting that the pleiotropic phenotypes of mfa-1ΔRIP null strains resulted from mutations outside the mfa-1 ORF. It is of interest that disruption of the 3′ UTR of mfp (an mfa-1 homologue) results in reduced fertilizing activity in P. anserina (11), and downregulation of Mf2-2 (an mfa-1 homologue) by viral repression in C. parasitica leads to reduced asexual sporulation and impaired female fertility (51). Further study is needed to illuminate the precise function(s) of the 3′ UTR of mfa-1 during vegetative growth and development in N. crassa.

In a previous study, we showed that loss of pre-1, the mat A pheromone receptor gene, greatly affected expression of both pheromone precursor genes in unfertilized sexual tissues (20). Interestingly, expression of the pre-1 gene in unfertilized sexual tissues is reduced in the Δccg-4 background, although that of pre-2, the mat a pheromone receptor gene, was unaffected in the Δccg-4 or Δmfa-1 background. The reduction in pre-1 levels in a Δccg-4 mutant is unexpected, as there is currently no genetic evidence supporting the existence of a CCG-4 receptor in mat A cells. We have recently observed low but detectable levels of pre-2 expression in mat A females (unpublished observations). If translated, the small amount of PRE-2 produced may provide a receptor target for the CCG-4 pheromone, thus explaining the apparent regulation of pre-1 transcription by a pheromone response pathway transcriptional cascade.

Pheromones were initially shown to function in the early phases of the sexual cycle in fungi, during the processes of initial mate recognition and cell-cell fusion. However, recent studies have extended the involvement of pheromones beyond early cell fusion events. Pheromones are also known to be involved in meiosis in S. pombe (9), maintenance of the dikaryotic filamentous state in U. maydis (40), and nuclear migration and clamp cell fusion in basidiomycetes (reviewed in reference 7). A role for pheromones after fertilization is possible in N. crassa but cannot be extrapolated from our studies, due to the block in trichogyne attraction and cell fusion exhibited by the mfa-1 and ccg-4 null mutants.

Conidia engineered to express ccg-4 inappropriately in the mat a background (strain mfa-70) are able to attract trichogynes from protoperithecia of the same mating type (fl mat a) and to induce protoperithecial enlargement and melanization. However, beak formation and ascospore generation are absent from the fruiting bodies, indicating that fertilization has not occurred between the mfa-70 conidia and fl mat a trichogynes. When observed microscopically, the conidia and trichogynes display an unusual pattern of chemotropic response, support-
ing the absence of cell fusion. The multiple coiling events around different conidia exhibited by a single trichogyne may be the outcome of repeated stimulation and desensitization of the pheromone signaling pathway, due to the failure of cells of the same mating type to fuse. Results from numerous studies of S. cerevisiae demonstrate that when pheromones bind to mating-type-specific GPCRs on the cell surface, the signal is transduced to the cytoplasmic compartment through interaction with a G protein, subsequently changing the pattern of gene expression to arrest the cell in the G1 phase and to form a mating projection toward the source of the pheromone (reviewed in reference 13). However, prolonged pheromone stimulation downregulates the signaling pathway by inducing expression of proteases that destroy the pheromones, modifying enzymes to facilitate internalization of the pheromone receptors and modifying the RGS (regulator of G-protein signaling) protein Sst2p to inactivate Gpa1p (Go) (13). Thus, despite the development of profound morphological changes, cells that fail to mate eventually become refractory to pheromone stimulation and resume cell division. A similar mechanism may explain the aberrant chemotropic behavior of N. crassa trichogynes when presented with conidia of the same mating type that are expressing the pheromone of the opposite mating type.

Taken together, these data support the following model for early events during mating in N. crassa. First, pheromones and pheromone receptors are in fact initial determinants for sexual identity and compatibility during mate recognition. Second, pheromone signaling can be transmitted between cells of the same mating type if they are suitably paired for the expression of a pheromone and its cognate receptor. Third, additional determinants, such as the mating-type genes matA and mata, are required for normal cell fusion between gametes of the opposite mating type, likely because they are essential for subsequent nuclear fusion and meiosis. The latter may explain why coexpression of ceg-4 and pre-2 in a single strain failed to induce self-fertility in N. crassa. Future experiments will probe the detailed requirements for pheromones and mating-type genes during sexual reproduction in this heterothallic species.

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