Roles for Receptors, Pheromones, G Proteins, and Mating Type Genes During Sexual Reproduction in *Neurospora crassa*

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ABSTRACT Here we characterize the relationship between the PRE-2 pheromone receptor and its ligand, CCG-4, and the general requirements for receptors, pheromones, G proteins, and mating type genes during fusion of opposite mating-type cells and sexual sporulation in the multicellular fungus *Neurospora crassa*. PRE-2 is highly expressed in *mat a* cells and is localized in male and female reproductive structures. Δ*pre-2 mat a* females do not respond chemotropically to *mat A* males (conidia) or form mature fruiting bodies (perithecia) or meiotic progeny (ascospores). Strains with swapped identity due to heterologous expression of *pre-2* or *ccg-4* behave normally in crosses with opposite mating-type strains. Coexpression of *pre-2* and *ccg-4* in the *mat A* background leads to self-attraction and development of barren perithecia without ascospores. Further perithecial development is achieved by inactivation of *Sad-1*, a gene required for meiotic gene silencing. Findings from studies involving forced heterokaryons of opposite mating-type strains show that presence of one receptor and its compatible pheromone is necessary and sufficient for perithecial development and ascospore production. Taken together, the results demonstrate that although receptors and pheromones control sexual identity, the mating-type genes (*mat A* and *mat a*) must be in two different nuclei to allow meiosis and sexual sporulation to occur.

DISTINCTION between self and nonself is a fundamental requirement for various biological events in eukaryotes, ranging from somatic growth and mate selection in fungi (Janeway and Medzhitov 2002; Aanen *et al.* 2010; Casselton and Feldbrugge 2010; Debuchy *et al.* 2010) to immune defense in vertebrates (Janeway and Medzhitov 2002; Boehm 2006). In heterothallic ascomycete filamentous fungi, non–self-recognition is important during vegetative growth and sexual reproduction (see reviews in Glass and Dementhon 2006; Aanen *et al.* 2010). During vegetative growth, fusions between cells are constantly formed, generating a network of interconnected hyphae that is important for cell–cell communication and homeostasis in an individual colony. Hyphal fusion between individuals that differ at certain loci results in rejection of heterokaryon formation, leading to programmed death of the fusion cell. This process is referred to as heterokaryon incompatibility and is regulated by genetic differences at *het* loci (Glass and Dementhon 2006; Aanen *et al.* 2010).

As opposed to self-fertile homothallic species, which do not require a mating partner to complete sexual reproduction, heterothallic fungi are self-sterile and are only able to mate with a haploid cell of opposite mating type. The genetic barrier and sexual identity of heterothallic strains are established by the mating type (*mat*) genes. *mat* genes encode transcriptional regulators that control the expression of many genes required for sexual compatibility and reproduction, including the mating-type–specific pheromone and its G-protein–linked receptor (reviewed by Debuchy *et al.* 2010). The mating process of heterothallic fungi is best understood in the yeast *Saccharomyces cerevisiae* (Kurjan 1992; Bardwell 2005). Morphologically identical haploid cells of opposite mating type (*MAT***a** and *MAT*α) mutually stimulate

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each other by secreting small peptide pheromones that are detected by cell-surface receptors. The pheromone–receptor interaction activates a mitogen activated protein kinase (MAPK) cascade, triggering several cellular responses, including cell cycle arrest in G1, elongation toward cells of opposite mating type, and transcription of genes involved in cellular and nuclear fusion. Under appropriate environmental conditions, the resulting diploid cell then undergoes meiosis to form four haploid spores (Cross *et al.* 1988; Herskowitz 1988).

Neurospora crassa is a heterothallic filamentous fungus with two mating types, *mat a* and *mat A*. *N. crassa* undergoes a more complex process of asexual and sexual reproduction than unicellular yeasts (Raju 1992b; Springer 1993). During vegetative growth, *N. crassa* produces tubular structures called hyphae and two types of mitotic spores: multicellular macroconidia and uninucleate microconidia. Under nitrogen starvation, the vegetative hyphae undergo complex morphological changes to form a spherical multicellular female reproductive structure (protoperithecium), which extends a female-specific receptive hypha (trichogyne) (Raju 1992b). A trichogyne of one mating type is attracted to and fuses with a male cell of opposite mating type (a macroconidium, microconidium or hyphal fragment). After fusion between the trichogyne and conidium, the male nucleus is transported to the base of the protoperithecium where nuclei of opposite mating type recognize each other and undergo synchronous rounds of mitosis. The male and female nuclei then fuse and the diploid zygote nucleus immediately enters meiosis. During this process, the protoperithecium enlarges greatly to become the flask-shaped perithecium. The haploid meiotic products undergo a round of postmeiotic mitosis and are then packaged into a spore (ascospore) in an eight-spored ascus. When fully mature, the ascospores are ejected through a pore (ostiole) at the tip of the perithecium.

In recent years, our laboratory has demonstrated that the pheromone receptor gene expressed in *mat A* cells, *pre-1*, is required for *mat A* trichogynes to recognize and fuse with *mat a* male cells (Kim and Borkovich 2004) . The *ccg-4* and *mfa-1* pheromone genes are necessary for chemotropic attraction and male fertility in *mat A* and *mat a* strains, respectively, with the prediction that PRE-1 binds to MFA-1, while PRE-2 recognizes CCG-4 (Bobrowicz *et al.* 2002; Kim *et al.* 2002; Kim and Borkovich 2006). Similar to Δ*pre-1 mat A* strains, trichogynes of Δ*gna-1* (Gα), Δ*gnb-1* (Gβ), and Δ*gng-1* (Gγ) mutants are completely unresponsive to male cells of opposite mating type (Kim and Borkovich 2004; Krystofova and Borkovich 2005). In particular, data support coupling of the Gα subunit GNA-1 to pheromone receptors and transduction of the pheromone signal (Kim and Borkovich 2004). Finally, we have shown that although coexpression of a compatible pheromone and receptor in the same strain triggers self-stimulation, only abortive perithecia with no ascospores are produced, suggesting additional requirements for successful sexual reproduction in *N. crassa* (Kim and Borkovich 2006).

In this study, we generated and characterized mutants lacking *pre-2*, a gene that encodes the pheromone receptor expressed in *mat a* cells. Mutants lacking both pheromone receptor genes, *pre-1* and *pre-2*, were also analyzed. We crafted strains with swapped identity through high-level, heterologous expression of *pre-2* or *ccg-4* (the PRE-2 pheromone) in the absence or presence of both mating-type genes as well as in a strain background that eliminates meiotic silencing. We also determined the minimum requirement for receptors, pheromones, and the Gα gene *gna-1* during sexual reproduction in forced heterokaryons between opposite mating type strains. Our data indicate that pheromones and receptors are important for initial recognition of mates. However, additional determinants, such as the presence of *mat A* and *mat a* genes in two different nuclei, are required for nuclear fusion, meiosis, and ascospore production.

Materials and Methods

Strains and growth conditions

The *N. crassa* strains used in this study are listed in Table 1. The strains were grown either on Vogel's minimal medium (VM) (Vogel 1964) for vegetative growth or on synthetic crossing medium (SCM) (Westergaard and Mitchell 1947) to induce sexual development. Sorbose-containing medium [Fructose-Glucose-Sorbose (FGS)] (Davis and Deserres 1970) was used to facilitate colony formation on plates. Supplements were added for auxotrophic strains as necessary and hygromycin B was used at 200 μg/ml in media where indicated. Conidia from 7-day-old VM agar cultures were used for inoculation of cultures. For submerged cultures, conidia were inoculated in liquid VM at 3×10^6 cells/ml and grown in the dark at 30° for 5, 16, or 24 hr with agitation. For differentiated cultures on solid medium, 1 μl of a conidial suspension was inoculated on the center of VM or SCM plates. VM plate cultures were grown in the dark at 30° for 3 days, while SCM plate cultures were grown for 6 days at 25° under constant light. For growth of cultures for RNA or protein extractions, plates were overlaid with cellophane (Bio-Rad Laboratories, Hercules, CA), and differentiated tissues were collected from the plates and frozen immediately in liquid nitrogen. For perithecial tissues, strains were grown on SCM plates and fertilized with a conidial suspension of opposite mating type. Sexual tissues were scraped from the plates 3 or 6 days after fertilization and frozen immediately. All plasmids were maintained in *Escherichia coli* strain DH5α (Hanahan 1983).

Isolation of Δpre-2 and Δpre-2::hph, pre-2$^+$ complemented strains

A construct (pHK15) was generated to replace the 1.7-kb *pre-2* open reading frame (ORF) with the 1.4-kb *hph* cassette encoding *E. coli* hygromycin B phosphotransferase under the control of the *Aspergillus nidulans trpC* promoter (pCSN44) (Staben *et al.* 1989). The 1.2-kb 5′ and 3.2-kb 3′ flanking DNAs for the *pre-2* ORF were amplified from a genomic cosmid clone (G18A5) (Orbach 1994) in polymerase chain reactions (PCRs). Primers 1 and 2 were used for the 5′

Table 1 ***N. crassa*** **strains**

Strain	Relevant genotype	Comments	Source or reference
74A; FGSC 987	74-OR23-1A	Wild type	FGSC
74a; FGSC 988	74-OR-1a	Wild type	FGSC
A^{m44}; FGSC 4570	*un-3 ad-3A nic-2 cyh-1 A*^{m44}	*mat A* sterile mutant	FGSC
a^{m1}; FGSC 4564	*ad-3B cyh-1 a*^{m1}	*mat a* sterile mutant	FGSC
a^{m33}; FGSC 5382	*a*^{m33}	*mat a* mutant	FGSC
FGSC 4317	*fl mat A*		FGSC
FGSC 4347	*fl mat a*		FGSC
FGSC 8740	Δ*Sad-1*::*hph mat A*		FGSC
FGSC 8741	Δ*Sad-1*::*hph mat a*		FGSC
FGSC 6103	*his-3 mat A*		FGSC
his3a	*his-3 mat a*		Krystofova and Borkovich (2005)
pan-2 A	*pan-2 mat A*		R. L. Weiss (UCLA)
pan-2 a	*pan-2 mat a*		Kim and Borkovich (2006)
16A	Δ*pre-1*::*hph mat A*		Kim and Borkovich (2004)
16a	Δ*pre-1*::*hph mat a*		Kim and Borkovich (2004)
16Ahis3	Δ*pre-1*::*hph his-3 mat A*		Kim and Borkovich (2004)
16ahis3	Δ*pre-1*::*hph his-3 mat a*		Kim and Borkovich (2004)
16Apan2	Δ*pre-1*::*hph pan-2 mat A*		Kim and Borkovich (2004)
16a pan2	Δ*pre-1*::*hph pan-2 mat a*		Kim and Borkovich (2004)
c1a	Δ*ccg-4*::*hph mat a*		Kim and Borkovich (2006)
c1ahis3	Δ*ccg-4*::*hph his-3 mat a*		Kim and Borkovich (2006)
c1apan2	Δ*ccg-4*::*hph pan-2 mat a*		Kim and Borkovich (2006)
m1a70	Δ*mfa-1*::*hph* P*ccg-1*::*ccg-4*$^{+}$::*his-3*$^{+}$ *mat a*		Kim and Borkovich (2006)
1B4	Δ*gna-1*::*hph mat A*		Ivey *et al.* (1999)
B3	Δ*gna-1*::*hph* Δ*gna-2*::*pyrG*$^{+}$ *mat a*		Kays and Borkovich (2004)
Δ1his3	Δ*gna-1*::*hph his-3 mat A*	Progeny of FGSC 6103 × B3	This study
Δ1pan2	Δ*gna-1*::*hph pan-2 mat a*	Progeny of pan-2 A × B3	This study
23	Δ*pre-2*::*hph mat A* heterokaryon	74A pHK15 transformant	This study
32	Δ*pre-2*::*hph mat A* heterokaryon	74A pHK15 transformant	This study
23A	Δ*pre-2*::*hph mat A*	Progeny of 23 × 74a	This study
23a	Δ*pre-2*::*hph mat a*	Progeny of 23 × 74a	This study
32A	Δ*pre-2*::*hph mat A*	Progeny of 32 × 74a	This study
32a	Δ*pre-2*::*hph mat a*	Progeny of 32 × 74a	This study
23Ahis3	Δ*pre-2*::*hph his-3 mat A*	Progeny of 23a × FGSC 6103	This study
23ahis3	Δ*pre-2*::*hph his-3 mat a*	Progeny of 23a × FGSC 6103	This study
32ahis3	Δ*pre-2*::*hph his-3 mat a*	Progeny of 32a x FGSC 6103	This study
23Apan2	Δ*pre-2*::*hph pan-2*, *mat A*	Progeny of 23a × *pan-2 mat A*	This study
23apan2	Δ*pre-2*::*hph pan-2*, *mat a*	Progeny of 23a × *pan-2 mat A*	This study
23-1	Δ*pre-2*::*hph his-3*$^{+}$::*pre-2*$^{+}$ *mat a*	23ahis3 pHK12 transformant	This study
32-4	Δ*pre-2*::*hph his-3*$^{+}$::*pre-2*$^{+}$ *mat a*	32ahis3 pHK12 transformant	This study
P1P2A	Δ*pre-1*::*hph* Δ*pre-2*::*hph mat A*	Progeny of 16a × 23A	This study
P1P2a	Δ*pre-1*::*hph* Δ*pre-2*::*hph mat a*	Progeny of 16a × 23A	This study
P1P2h3A	Δ*pre-1*::*hph* Δ*pre-2*::*hph his-3 mat A*	Progeny of 16a × 23Ahis3	This study
P1P2h3a	Δ*pre-1*::*hph*, Δ*pre-2*::*hph his-3 mat a*	Progeny of 16a × 23Ahis3	This study
P1P2pan2A	Δ*pre-1*::*hph*, Δ*pre-2*::*hph pan-2 mat A*	Progeny of 16a × 23Apan2	This study
P1P2pan2a	Δ*pre-1*::*hph* Δ*pre-2*::*hph pan-2*, *mat a*	Progeny of 16a × 23Apan2	This study
P1A17	Δ*pre-1*::*hph* P*ccg-1*::*pre-2-GFP*::*his-3*$^{+}$ *mat A*	16Ahis3 pHK17 transformant	This study
P2a17	Δ*pre-2*::*hph* P*ccg-1*::*pre-2-GFP*::*his-3*$^{+}$ *mat a*	23ahis3 pHK17 transformant	This study
P1A35	Δ*pre-1*::*hph* P*pre-1*::*pre-2-GFP*::*his-3*$^{+}$ *mat A*	16Ahis3 pHK35 transformant	This study
P1A46	Δ*pre-1*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat A*	16Ahis3 pHK46 transformant	This study
P2a46	Δ*pre-2*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat a*	23ahis3 pHK46 transformant	This study
P1Gα1h3A	Δ*pre-1*::*hph* Δ*gna-1*::*hph his-3 mat A*	16ahis3 × 1B4 progeny	This study
P1Gα1A46	Δ*pre-1*::*hph* Δ*gna-1*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat A*	P1Gα1AH3 pHK46 transformant	This study
P1C4a46	Δ*pre-1*::*hph* Δ*ccg-4*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat a*	Progeny of c1a × P1A46	This study
P1C4A46	Δ*pre-1*::*hph* Δ*ccg-4*::hph P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat A*	Progeny of P1C4a46 × P1A46	This study
P1A46-68	Δ*pre-1*::*hph*, P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$ *mat a-1*$^{+}$::*bar mat A*	P1A46 pHK68 transformant	This study
P1A68	Δ*pre-1*::*hph mat a-1*$^{+}$::*bar*$^{+}$ *mat A*	Progeny of FGSC 8741 × P1A46-68	This study

(continued)

Table 1, *continued*

Strain	Relevant genotype	Comments	Source or reference
P1P2C4A116	Δ*pre-1*::*hph* Δ*pre-2*::*hph* Δ*ccg-4*::*hph mat A*	Progeny of c1apan2 × P1P2pan2A	This study
P1P2C4a48	Δ*pre-1*::*hph* Δ*pre-2*::*hph* Δ*ccg-4*::*hph mat a*	Progeny of c1apan2 × P1P2pan2A	This study
P1A46-Sad1	Δ*pre-1*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^+$ Δ*Sad-1*::*hph mat A*	Progeny of FGSC 8741 × P1A46-68	This study
P1A68-Sad1	Δ*pre-1*::*hph mat a-1*$^+$::*bar* Δ*Sad-1*::*hph mat A*	Progeny of FGSC 8741 × P1A46-68	This study
P1A46-68-Sad1	Δ*pre-1*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^+$ *mat a-1*$^+$::*bar* Δ*Sad-1*::*hph mat A*	Progeny of FGSC 8741 × P1A46-68	This study

FGSC, Fungal Genetics Stock Center, Kansas City, MO.

flanking DNA and primers 3 and 4 were for the 3′ flanking DNA (Table 2). To facilitate the cloning process, *Bam*HI, *Hin*dIII, and *Spe*I restriction enzyme sites were introduced into primers 2, 3, and 4, respectively. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI), yielding pHK13 (5′) and pHK14 (3′). The 1.4-kb *hph* cassette was released from pCSN44 using *Bam*HI and *Hin*dIII, and pHK14 was digested with *Hin*dIII and *Spe*I to excise the 3′ flanking DNA. Finally, pHK13 (containing the 5′ flank) was linearized using *Bam*HI and *Spe*I and the *hph* cassette and the 3′ flank were inserted, yielding pHK15.

pHK15 (2 μg) was linearized with *Spe*I and used to electroporate wild-type *N. crassa* strain 74A as described (Ivey *et al.* 1996). Transformants were isolated by plating on FGS plates containing hygromycin. Genomic DNA was isolated using the Puregene DNA kit (Gentra Systems, Minneapolis, MN) and subjected to Southern analysis after digestion with *Bam*HI. Transformants with the Δ*pre-2* mutation were identified by using the 1.9-kb *Nco*I fragment from pHK7 as a probe. pHK7 was constructed by PCR-amplifying (using primers 5 and 6; Table 2) and cloning a 3.5-kb 5′ flanking DNA for the *pre-2* ORF into the pGEM-T vector. All probes were labeled using the Prime-a-Gene labeling system (Promega).

Two heterokaryotic Δ*pre-2* strains (strains 23 and 32; Table 1) were selected and crossed to wild-type strain 74a, and progeny were selected on FGS hygromycin plates. Southern analysis was used to verify homokaryotic Δ*pre-2*::*hph* strains (data not shown). The homokaryons were subsequently crossed to *his-3* or *pan-2* strains of opposite mating type (see Table 1) to obtain auxotrophic Δ*pre-2*::*hph* strains. To isolate prototrophic and auxotrophic Δ*pre-1* Δ*pre-2* double mutants, the Δ*pre-2 his-3* and Δ*pre-2 pan-2* strains were individually crossed to Δ*pre-1*::*hph* strains of opposite mating type (Kim and Borkovich 2004; Table 1).

The *pre-2* complementation construct pHK12 was generated by cloning a 4.4-kb wild-type *pre-2*$^+$ fragment into the *Xba*I site of the *his-3* targeting vector pRAUW122 (Aramayo and Metzenberg 1996). The *pre-2*$^+$ genomic fragment extends 1.9 kb upstream to 0.8 kb downstream of the *pre-2* ORF and was amplified from the G18A5 cosmid in PCRs using primers 7 and 8, both of which contained an introduced *Xba*I site to facilitate the cloning process (Table 2). pHK12 was electroporated into Δ*pre-2* mutant strains 23ahis3 and 32ahis3, and transformants were selected on FGS plates with no added histidine. Heterokaryotic transformants that contained the desired integration event at the *his-3* locus were identified by Southern analysis of genomic DNA, using the 8.8-kb *Hin*dIII fragment from pRAUW122 as a probe. All genomic DNAs were digested with *Hin*dIII and *Bam*HI. Homokaryons were isolated using the microconidiation procedure as described (Ebbole and Sachs 1990) and verified by Southern analysis.

Northern analysis

For Northern analysis, total RNA was isolated using the Purescript RNA isolation kit (Gentra Systems) following the manufacturer's instructions. Samples containing 10 or 30 μg of total RNA were subjected to Northern analysis (Tsui *et al.* 1994). All probe templates were labeled using the random primer method as described above. For the *pre-2* probe, the entire 1.7-kb ORF was amplified from the G18A5 cosmid using primers 9 and 10 (Table 2). For the *mat a-1* probe, the gene was excised from pCSN4 as a 1.9-kb *Xho*I–*Eco*RI fragment (Staben *et al.* 1989). The *pre-1*, *ccg-4*, and *mfa-1* probes were prepared as described previously (Kim and Borkovich 2004, 2006).

Expression and localization of PRE-2–FLAG and PRE-2–GFP fusion proteins

Expression of a C-terminal FLAG-epitope–tagged version of PRE-2 was driven by the highly expressed and mating-type–independent *ccg-1* promoter (P*ccg-1*) (Loros *et al.* 1989). The construct was cloned into pHK40, a *his-3* targeting vector (Kim and Borkovich 2006). The P*ccg-1*::*pre-2* fragment was amplified from pHK17 (see below) by PCR using primers 11 and 12. Primers 11 and 12 each contained a *Sma*I site to facilitate the cloning process (Table 2) and primer 12 was also designed with a 24-bp sequence encoding the FLAG epitope (DYKDDDDK) (Brizzard *et al.* 1994) followed by a stop codon (TAG; Table 2). The resulting P*ccg-1*::*pre-2-FLAG* fragment was then digested with *Sma*I and inserted into pHK40 cut with *Xba*I and then made blunt using Klenow. A Δ*pre-2 his-3 mat a* strain (23ahis3; Table 1) was electroporated with pHK46, and transformants with the desired integration of pHK46 at the *his-3* locus were selected for histidine prototrophy and verified by Southern analysis. All genomic DNAs were digested with *Hin*dIII and the 8.8-kb *Hin*dIII fragment from pRAUW122 was used as a probe.

Table 2 Oligonucleotides used in this study

Name	Sequence (5′–3′)
1	TACATCCGCATACCTGCCTTCATA
2	GTGTTGATGGGATCCCCAATGAAT
3	GTGCTCATGTCCATGAAGCTTG
4	GTGCTGGTGGTAGCACTAGTTA
5	TCACTGGCTGGGCTACTATTACAG
6	GATGTGTGGAAGGGATCTAGAGCT
7	GAGGATTCTAGACAGTGTGGGAGA
8	CATCGAATCTAGACCGTACGCACC
9	ATCTTCAGCGGCATAACCCA
10	TCAAAAGACCTCGGCTTCGT
11	GGCCCGGGTAGAAGGAGCAGTCCATCTGCGTG
12	GCCCGGGCTACTTATCGTCGTCATCCTTGTAATCCTCAAA AGACCTCGGCTTCGTGAC
13	TTTCTAGATCACCCGTGCATC
14	CCAGATGGATCCATGTGATGG
15	ATCGTGCGGCCGCTGACTATACAC
16	GGTGTACTAGTAACTCGCGAAAGTCCG
17	CCACCACCATGCTGAGGATCTCGGG
18	GTTACCTAACCATCCACCCTTGTCTTGTCC
19	CTCCACTAGCTCCAGCCAAGCCC
20	CACCATCTCCCACGCTTACTGCATATTAGGG

Genomic DNA of heterokaryotic transformants contained 9.5-kb, 8.2-kb, and 3.9-kb hybridizing fragments while that of the Δ*pre-2* recipient strain contained only a 9.5-kb hybridizing fragment (data not shown). Homokaryons were isolated using the microconidiation method (Ebbole and Sachs 1990) and verified by Southern analysis. Strain P2a46 (Table 1) was chosen for further analysis. The P1A46 strain (Table 1) was isolated by transformation of Δ*pre-1 mat A* strain 16A using the procedures described for P2a46, above.

Immunoprecipitation of the PRE-2–FLAG protein was performed as described with minor modifications (Krystofova and Borkovich 2005). Whole cell extracts were prepared from P2a46 and wild-type strains and immunoprecipitated protein samples were resolved on a 10% SDS–PAGE gel. The gel was blotted onto a PVDF membrane (Millipore) and subjected to Western analysis. A mouse anti-FLAG antibody (Sigma; 1:2000) was used as the primary antibody and a HRP-conjugated goat antimouse IgG (Bio-Rad; 1:10,000) was used as the secondary antibody. Immunoreactive proteins were detected by chemiluminescence (ECL; Pierce, Rockford, IL) with a Biochemi system (UVP BioImaging Systems, Upland, CA). A duplicate gel was Coomassie stained to verify equal protein loading (Sambrook and Russell 2001).

The *his-3* targeting vector pMF272 has been used for overexpression of green fluorescent protein (GFP) fusion proteins under the control of the *N. crassa ccg-1* promoter (Folco *et al.* 2003). For overexpression of PRE-2–GFP, the entire *pre-2* ORF was amplified from the G18A5 cosmid in PCRs using primers 13 and 14. To facilitate cloning, *Xba*I and *Bam*HI sites were introduced into primers 13 and 14, respectively. pMF272 was digested with *Xba*I and *Bam*HI, creating an opening between P*ccg-1* and the *GFP* gene. The *pre-2* PCR product was digested using *Xba*I and *Bam*HI and ligated with *Xba*I–*Bam*HI-digested pMF272 to yield pHK17. The stop codon (TAG) in the *pre-2* ORF was replaced with a Trp codon (TAT) to create the carboxyterminal translational fusion of *pre-2* and *GFP* (Table 2).

Plasmid pHK35 was constructed to express the PRE-2–GFP fusion protein under the control of the mating-type–specific *pre-1* promoter in *mat A* strains. The *pre-1* promoter region was amplified as a 2-kb fragment from pHK6, the *pre-1* rescue construct (Kim and Borkovich 2004), by PCR using primers 15 and 16. Primers 15 and 16 contain *Not*I and *Spe*I sites, respectively (Table 2). The 2-kb PCR product was digested with *Not*I and *Spe*I and cloned into *Not*I–*Xba*I-digested pHK17 as a replacement of the *ccg-1* promoter, yielding pHK35.

pHK17 and pHK35 were separately electroporated into Δ*pre-2 his-3 mat a* strain 23ahis3 and Δ*pre-1 his-3 mat A* strain 16Ahis3, respectively. Transformants with the desired integration of pHK17 or pHK35 at the *his-3* locus were isolated on the basis of histidine prototrophy and confirmed by Southern analysis. All genomic DNAs were digested with *Hin*dIII and blots probed with the 5.2 kb *Hin*dIII–*Bam*HI fragment from pRAUW122. When subjected to Southern analysis, genomic DNA from all heterokaryotic pHK17 or pHK35 transformants contained a 9.5-kb hybridizing fragment corresponding to the wild-type gene (data not shown). In addition, pHK17 transformants contained a 5.2-kb fragment, while those from pHK35 possessed 4.5- and 3.9-kb fragments. Homokaryotic pHK17 (strain P2a17) or pHK35 (strain P1A35) strains obtained using the microconidiation method (see above) did not contain the 9.5-kb wild-type fragment.

Western analysis was used to verify expression of the PRE-2–GFP fusion protein. Whole cell extracts (Turner and Borkovich 1993) were prepared from liquid VM submerged or SCM plate cultures of the P2a17 and wild-type strains and protein concentrations were determined as described above. Samples containing 15 μg of total protein were resolved on 7.5% SDS–PAGE gels and transferred onto PVDF membranes (Millipore). Blots were probed with a rabbit anti-GFP antibody (Abcam, Cambridge, MA; 1:2000 dilution) followed by a horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (Bio-Rad; 1:10,000 dilution), as recommended by the manufacturer (Abcam). Detection of immunoreactive proteins was as described above for PRE-2–FLAG.

To determine the tissue localization of the PRE-2–GFP protein, strains were grown on 2% water agar plates and observed microscopically as described (Kim and Borkovich 2004). Protoperithecia were visualized using a Leica TCS SP2 laser-scanning confocal microscope (Leica, Wetzlar, Germany) at the University of California Riverside Core Instrumentation Facility, while trichogynes and conidia were imaged using a BX41 fluorescent microscope with UM Plan Fluorite objective lenses and a PM-C35B camera (Olympus America, Lake Success, NY).

Generation of self-stimulating strains

For coexpression of *ccg-4* and its receptor, *pre-2*, in the same strain, pHK46 was transformed into Δ*pre-1 his-3 mat A* strain

16Ahis3. Isolation and purification of Δ*pre-1 Pccg-1*::*pre-2*::*his-3 mat A* transformants using the microconidiation procedure (representative strain P1A46; Table 1) was as described above. The involvement of GNA-1 (Gα) and CCG-4 in self-stimulation was analyzed by introducing the Δ*gna-1* and Δ*ccg-4* mutations into the P1A46 strain background. For the Δ*gna-1* strain, a Δ*pre-1 his-3 mat a* female was crossed with a Δ*gna-1 mat A* male, and histidine-requiring hygromycin-resistant progeny were selected. The presence of the Δ*pre-1* and Δ*gna-1* mutations was verified by Southern analysis as described previously (Ivey *et al.* 1996; Kim and Borkovich 2004). A Δ*pre-1* Δ*gna-1 his-3 mat A* strain (P1Gα1h3A; Table 1) was then electroporated with pHK46. Transformants with pHK46 integrated at the *his-3* locus were isolated, verified, and purified using the microconidiation procedure as described above. Representative strain P1Gα1A46 was chosen for further analysis.

Strains lacking *ccg-4* were isolated in both mating types in the P1A46 background. To generate a *mat a* strain lacking *ccg-4*, P1A46 was crossed as a male to a Δ*ccg-4 mat a* strain c1a as a female (Table 1). Hygromycin-resistant progeny were selected, tested for mating type, and genomic DNAs were subjected to Southern analysis to determine Δ*pre-1*, ectopic *pre-2* (integrated at *his-3* locus), and Δ*ccg-4* genotypes (see above; Kim and Borkovich 2006). Δ*pre-1* Δ*ccg-4 Pccg-1*::*pre-2*::*his-3 mat a* strain P1C4a46 was chosen for further analysis. To isolate a *mat A* strain, P1C4a46 was crossed as the female parent to strain P1A46 as the male. Progeny were isolated, tested for mating type, and the presence of the Δ*ccg-4* mutation was verified by Southern analysis (Kim and Borkovich 2006). Representative strain P1C4A46 was subjected to phenotypic analysis.

Effects of the mat a-1 and Sad-1 genes in self-stimulating strains

To express the *mat a-1* gene in the *mat A* background, pHK68 was constructed as follows. The *mat a-1*$^+$ gene was obtained from pCSN4 as a 1.9-kb *Xho*I–*Eco*RI fragment (Staben *et al.* 1989) and ligated into *Xho*I–*Eco*RI digested pTJK1. pTJK1 is a pBluescript-based plasmid carrying the *bar*$^+$ phosphinothricin-resistance gene (Pall and Brunelli 1994; Jones *et al.* 2007). The resulting construct pHK68 was then transformed into P1A46, a strain that inappropriately expresses *pre-2* in the *mat A* background (Table 1). Transformants were isolated on FGS plates containing 200 μg/ml phosphinothricin and 0.5% proline as the sole nitrogen source (Pall and Brunelli 1994). The presence of the full-length ectopic *mat a-1* gene in these transformants was verified by Southern analysis. Genomic DNAs were digested with *Xho*I and *Eco*RI and blots were probed with the 1.9-kb *mat a-1* fragment. Representative strain P1A46-68 was chosen for phenotypic analysis (Table 1).

A possible influence of the *Sad-1* gene (Shiu *et al.* 2001) on expression of the ectopic *pre-2* and *mat a-1* genes in the P1A46 background was investigated. Δ*Sad-1 mat a* strain FGSC 8741 was crossed as a female to strain P1A46-68 (Δ*pre-1*::*hph* P*ccg-1*::*pre-2-FLAG*::*his-3*$^+$ *mat a-1*$^+$::*bar*$^+$ *mat A*) as a male (Table 1). Progeny were screened by plating on FGS medium ± hygromycin to isolate all possible combinations of genotypes. All isolates were then spot tested to check for resistance to phosphinothricin. The hygromycin- and/or phospinothricin-resistant progeny were examined for the presence of the Δ*pre-1*, Δ*Sad-1*, *pre-2*^{ec}, and *mat a-1*^{ec} alleles using Southern analysis (see above; Shiu *et al.* 2001). Expression of *pre-2* and *mat-a1* was determined using Northern analysis, as described above.

Phenotypic analysis '

Growth rate, hyperosmotic sensitivity, conidiation, heterokaryon formation, and CAT fusion assays: Apical extension rates, hyperosmotic sensitivity, morphology of submerged cultures, and growth in standing liquid cultures were examined as previously described (Ivey *et al.* 1996; Kays *et al.* 2000; Kim and Borkovich 2004). Formation and fusion of conidial anastomosis tubes were assayed (Fleissner *et al.* 2005; Kim and Borkovich 2006). The ability of two strains to undergo hyphal fusion and form a heterokaryotic colony was tested (Davis and Deserres 1970; Kim and Borkovich 2004). For these experiments, the wild-type, a^{m1}, Δ*pre-2*, Δ*pre-1* Δ*pre-2*, and Δ*gna-1* strains contained different auxotrophic markers (*his-3*, *pan-2*, or *ad-3*; see Table 1).'

Fertility tests: Crosses between *N. crassa* strains were conducted using standard techniques and mating types of progeny from such crosses were determined using *fl mat a* and *fl mat A* females (Davis and Deserres 1970). To examine the involvement of the pheromone receptor genes during sexual development, Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains were crossed to wild type as either the female (protoperithecial) or male (fertilizing) parent to detect dominant mating-specific defects. The Δ*pre-2* and Δpre-1 Δ*pre-2* strains were crossed to sibling mutant strains to detect recessive traits for *pre-2* (alone or with *pre-1*) affecting sexual development.'

Trichogyne assay: Chemotropic responses of trichogynes to conidia of opposite mating type were examined as described previously (Bistis 1981; Kim and Borkovich 2004). Orientation and growth of trichogynes were monitored and photographed using a BX41 fluorescent microscope with UM Plan Fluorite objective lenses and a PM-C35B camera (Olympus America).'

Sexual development of forced heterokaryons between opposite mating-type strains: Forced heterokaryons formed between opposite mating type strains of various genotypes (see Figure 6) were examined for sexual development by cultivation on SCM plates. Each plate was prepared using 50 ml of medium for prolonged growth of heterokaryons. Formation of protoperithecia and development of perithecia were observed microscopically using a SZX9 stereomicroscope with an ACH 1X objective lens, and images were recorded using a PM-C35B camera (Olympus America).

Results

The pre-2 gene is preferentially expressed in mat a strains

To characterize the functions of the pheromone receptor *pre-2* in *N. crassa*, we generated Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains (see *Materials and Methods*). Replacement of the *pre-2* gene in all nuclei was confirmed by the correct hybridizing bands during Southern analysis and by the lack of *pre-2* transcript as assessed using Northern analysis (Figure 1A). Δ*pre-1* Δ*pre-2* double mutants were generated through sexual crosses between single mutants. The wild-type *pre-2*$^{+}$ genomic fragment was used to complement the Δ*pre-2* mutation. Confirmation that the *pre-2*$^{+}$ gene was successfully integrated at the *his-3* locus was achieved using Southern analysis and by observation of *pre-2* mRNA (Figure 1A; strains 23-1 and 32-4). Complemented strains exhibited wild-type phenotypes for all functions tested.

Our previous studies have shown that expression of the *mat A* pheromone receptor gene *pre-1* and the *ccg-4* and *mfa-1* pheromone precursor genes is mating-type specific and also developmentally regulated (Kim and Borkovich 2004, 2006). For example, *pre-1* is abundantly expressed in protoperithecia, perithecia, and conidia from *mat A* strains (Kim and Borkovich 2004). Therefore, we examined the mating type and developmental expression pattern of *pre-2* in *N. crassa*. Limiting nitrogen (SCM medium) stimulates development of female reproductive structures, protoperithecia. Fertilization of protoperithecia with male cells (usually conidia) of opposite mating type leads to perithecial development and sexual spore (ascospore) production. Northern analysis using RNA isolated from a *mat A* wild-type strain (74A) showed that *pre-2* mRNA is not expressed in vegetative cultures (data not shown) and that very low levels are present in SCM cultures producing protoperithecia (Figure 1B). In contrast, *pre-2* was expressed to relatively high levels in a *mat a* wild-type strain (74a). Amounts were highest in sexual tissue (protoperithecia), followed by conidia (0 hr) and germling (5 hr) cultures (Figure 1B). Lower levels were detected in older vegetative tissues (16 or 24 hr of submerged growth) and 3-day vegetative plate cultures (Figure 1B). High amounts of *pre-2* transcript were also detected in developing perithecia after fertilization with wild type. These results indicate that *pre-1* and *pre-2* share a similar pattern of developmental expression, but are regulated by different mating-type genes in *N. crassa* (Kim and Borkovich 2004, Figure 1B).

The mating-type–dependent expression of *pre-1* and *pre-2* was further analyzed using three previously characterized *N. crassa* mating-type mutants: *mat A* mutant A^{m44} and *mat a* mutants a^{m1} and a^{m33} (Griffiths and Delange 1978; Griffiths 1982). While A^{m44} and a^{m1} are both male and female sterile and do not produce pheromone (Bistis 1981; Bobrowicz *et al.* 2002; Kim *et al.* 2002), the a^{m33} mutant is fertile as a male or female and expresses both pheromones (Griffiths and Delange 1978; Bobrowicz *et al.* 2002). The major defect of a^{m33} is loss of mating-type incompatibility functions during vegetative growth. Consistent with pheromone receptor genes being regulated by mating type to control sexual fertility, *pre-1* and *pre-2* messages could not be detected in the A^{m44} and a^{m1} strains, respectively (Figure 1C; Kim and Borkovich 2004). In contrast, low but detectable levels of *pre-1* and *pre-2* are present in the a^{m33}strain that has retained sexual fertility functions (Figure 1C). These results support the mating-type specificity of pheromone receptor gene expression observed in wild-type strains and the requirement for pheromone receptors during mating in *N. crassa*.

In a previous study, we demonstrated that deletion of *pre-1* greatly affected expression of each pheromone precursor gene in the mating type that normally supports high message levels (Kim and Borkovich 2004). Therefore, we investigated the effect of *pre-2* on expression of *ccg-4* and *mfa-1*. We also determined whether loss of one pheromone receptor gene influences expression of the remaining receptor. As observed for Δ*pre-1* mutants (Kim and Borkovich 2004), deletion of *pre-2* alone or together with *pre-1* reduced expression of *ccg-4* to a great extent in the *mat A* background (Figure 1D). Similarly, *mfa-1* expression was significantly decreased in Δ*pre-1* and Δ*pre-2* single mutants and Δ*pre-1* Δ*pre-2* double mutants in the *mat a* mating type (Figure 1D). Surprisingly, *pre-1* message levels were greatly reduced in sexually differentiated tissues of the Δ*pre-2 mat A* strain and *pre-2* expression was drastically downregulated by the deletion of *pre-1* in the *mat a* background (Figure 1D). Taken together, these and our previous results demonstrate that loss of either pheromone receptor leads to reduced expression of both pheromones and the other pheromone receptor.

The PRE-2 protein is localized in protoperithecia, trichogynes, and conidia

Strains carrying a *pre-2* allele with a C-terminal FLAG epitope tag were generated in the Δ*pre-2* background (strain P2a46; Table 1). Expression of the *pre-2–FLAG* allele was driven by the highly expressed and mating-type–independent *ccg-1* promoter (Arpaia *et al.* 1995) to accumulate high levels of PRE-2–FLAG protein in either mating type. The FLAG epitope did not interfere with the function of the PRE-2 receptor, as the *pre-2–FLAG* allele complemented the defects of the Δ*pre-2* mutation. Immunoprecipitation of whole cell extracts from vegetative submerged (liquid VM) cultures with a FLAG antibody demonstrated that a reactive species of ~60 kDa could be detected in the P2a46 strain, but not in the untransformed wild-type control (Figure 2A). The size of the immunoreactive protein is in agreement with the molecular mass of PRE-2 (61.6 kDa) predicted using ExPASy Proteomics tools (http://web.expasy.org/compute_pi/). No PRE-2–FLAG could be immunoprecipitated from extracts obtained from P2a46 SCM plate cultures (Figure 2A). This result is consistent with rapid degradation of PRE-2 in sexually differentiated tissues, the site where pheromone receptors are assumed to be most active. Such a scenario is observed in *S. cerevisiae*, with ubiquitination leading to rapid turnover of pheromone receptors (Yin *et al.* 2005).

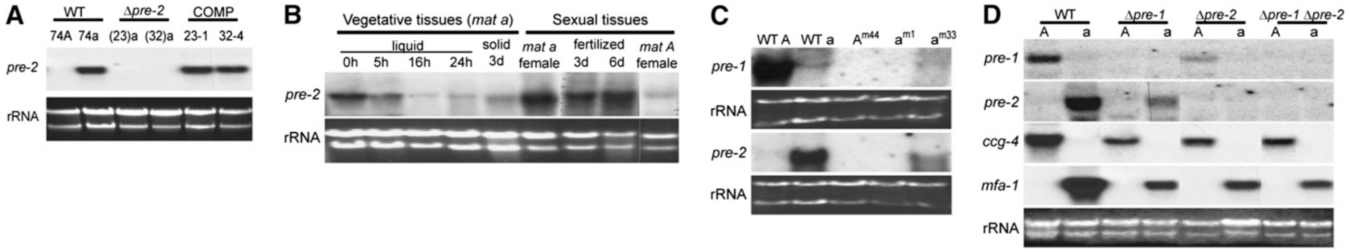

Figure 1 Northern analysis. (A) Verification of Δ*pre-2* and rescued strains. Total RNA was isolated from 6-day-old SCM plate cultures of *mat A* and *mat a* wild-type (WT) strains 74A and 74a; Δ*pre-2*::*hph* knockout mutants 23a and 32a (Δ*pre-2*), and Δ*pre-2*::*hph*$^{+}$, *pre-2*$^{+}$ complemented strains 23-1 and 32-4 (COMP). Approximately 20 μg of total RNA was subjected to Northern analysis using the *pre-2* ORF as a probe (see *Materials and Methods*). rRNA is the loading control. (B) *pre-2* mRNA levels in various tissues. Strains were wild-type *mat a* (74a) or *mat A* (74A), as indicated. RNA samples representing vegetative tissues were isolated from 74a grown under the following conditions: freshly harvested conidia (0 hr), conidia germinated in liquid VM for 5, 14, and 24 hr, and on solid VM plates for 3 days. RNA representing unfertilized sexual tissues was prepared from 74A or 74a grown on solid SCM plates for 6 days. RNA for fertilized sexual tissues was from perithecia harvested 3 and 6 days after fertilization, respectively, from a cross of 74a with 74A. Northern blots were probed as in A. rRNA is used as a loading control. (C) Expression of pheromone receptor genes in mating-type mutants. Total RNA was prepared from 6-day-old SCM plate cultures of wild-type *mat A* strain 74A (WT A), wild-type *mat a* strain 74a (WT a), the *mat A*^{m44} and *mat a*^{m1} sterile mutants, and the vegetative incompatibility mutant *mat a*^{m33}. Northern blots were probed using the *pre-2* (A) or *pre-1* ORF fragments (see *Materials and Methods*). rRNA is a loading control. (D) Pheromone receptor and pheromone precursor mRNA levels in strains lacking *pre-1* and/or *pre-2*. Strains are wild-type (WT) 74A (WT *mat A*), 74a (WT *mat a*), 16A (Δ*pre-1*, *mat A*), 16a (Δ*pre-1*, *mat a*), 23A (Δ*pre-2*, *mat A*), 23a (Δ*pre-2*, *mat a*), P1P2A (Δ*pre-1* Δ*pre-2*, *mat A*), and P1P2a (Δ*pre-*, Δ*pre-2*, *mat a*). Total RNA was isolated from 6-day-old SCM plate cultures. Northern blots were probed using *pre-1*, *pre-2*, *ccg-4*, and *mfa-1* specific probes (see *Materials and Methods*). rRNA is the loading control.

PRE-2–FLAG could be immunoprecipitated from solubilized plasma membrane but not cytosolic fractions, consistent with a plasma membrane localization for PRE-2 in *N. crassa* (data not shown). To further investigate the localization of PRE-2, we created a strain that expresses a PRE-2–GFP fusion protein under the control of the *ccg-1* promoter (strain P2a17; Table 1) in the Δ*pre-2* background. This construct complemented the defects of the Δ*pre-2* mutant (data not shown). Western analysis using a GFP-specific polyclonal antibody revealed a band of approximately 90 kDa was detected in submerged and SCM plate cultures of strain P2a17, but was absent from controls (Figure 2B). This size corresponds to GFP (29 kDa) plus the PRE-2 protein (61 kDa). In addition to the full-length PRE-2–GFP protein, lower molecular weight species were also detected (particularly in SCM plate cultures) of strain P2a17 (Figure 2B). These smaller proteins may result from rapid turnover of the PRE-2 protein in sexually differentiated tissue (see above).

We next analyzed the localization pattern of the PRE-2–GFP fusion protein in *N. crassa* tissues. Using a fluorescent compound microscope, the PRE-2–GFP protein was shown to be present in protoperithecia, trichogynes, and conidia (Figure 2C; data not shown). When protoperithecia were examined at higher resolution using a laser-scanning confocal microscope, PRE-2–GFP could only be detected in hyphae within the protoperithecium or in individual trichogynes extending from the structure (Figure 2C).

pre-2 has no apparent roles in vegetative functions or male fertility, but is essential for female fertility in mat a strains

Deletion of *pre-2* singly or in combination with *pre-1* did not cause any apparent defects during vegetative growth or development in either mating type. Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains displayed hyphal extension rates similar to those of wild-type strains on solid VM or SCM medium. Differentiation of aerial hyphae and conidia; sensitivity to elevated levels of NaCl, KCl, or sorbitol; and morphology in submerged cultures were also normal in Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains. Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains were able to undergo hyphal fusion and establish stable heterokaryons between same or opposite mating-type strains. Conidia of *pre-1* and *pre-2* single or double mutants also formed conidial anastomosis tubes (CATs) normally during germination and were able to fuse with neighboring conidia. Δ*pre-2* and Δ*pre-1* Δ*pre-2* strains were fertile as males in both mating types.

As mentioned above, mating in the heterothallic species *N. crassa* involves chemotropic interaction and fusion between female-specific hyphae (trichogynes) and male cells (typically conidia) of opposite mating type. This process is mediated by pheromones and pheromone receptors (Bistis 1981; Kim and Borkovich 2004, 2006). One way in which female fertility can be tested is by direct application of conidia of opposite mating type onto fully developed protoperithecia and subsequent assessment of perithecial development and ascospore production (Figure 3A; Davis and Deserres 1970). A second method investigates actual chemotropic interactions by assessing the ability of the trichogyne to track, coil around, and fuse with a conidium of opposite mating type (Figure 3B; Bistis 1981; Kim and Borkovich 2004, 2006). We used both of these assays to examine Δ*pre-2* and Δ*pre-1* Δ*pre-2* mutants for defects in trichogyne attraction and perithecial development. In crosses to wild-type *mat A* males, Δ*pre-2 mat a* strains were completely unable to develop perithecia, although they formed fully differentiated protoperithecia with trichogynes (Figure 3A). When examined microscopically, the trichogynes of Δ*pre-2 mat a* strains did not display chemotropic growth toward, coiling, or fusion with *mat A* male cells

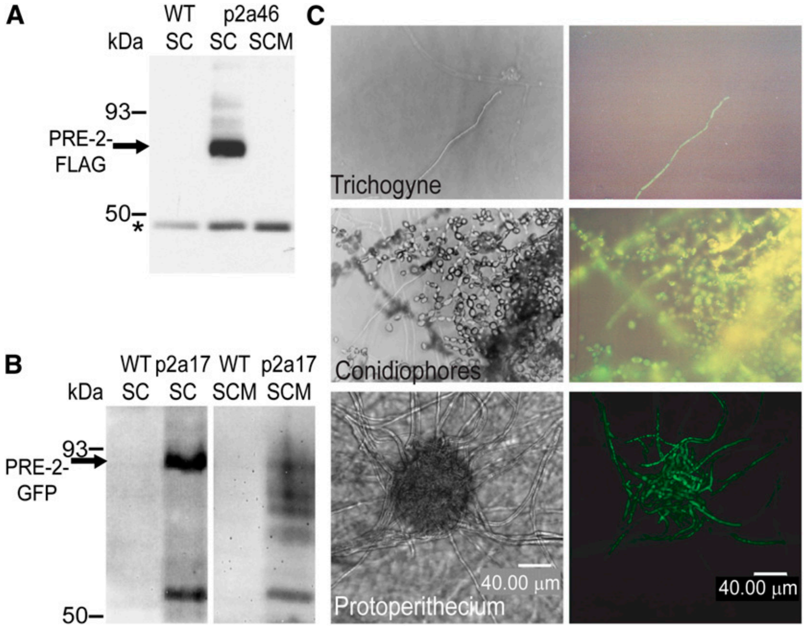

Figure 2 Expression and localization of the PRE-2 protein. (A) Immunoprecipitation and Western analysis of a PRE-2–FLAG fusion protein. Whole cell extracts were prepared from 16-hr submerged cultures (SC) of untransformed wild-type *mat a* strain 74a (WT) and a Δ*pre-2*::*hph*, P*ccg-1*::*pre-2-FLAG*::*his-3*$^+$ *mat a* strain (P2a46), and from sexually differentiated plate cultures (SCM) of strain P2a46. Samples containing 2 mg of total protein were immunoprecipitated using anti-FLAG M2-agarose (see *Materials and Methods*). Precipitated proteins were resolved on 10% SDS–PAGE gels and subjected to Western analysis using a FLAG-specific antiserum. The PRE-2–FLAG protein (∼62 kDa) is indicated by an arrow and a nonspecific band is shown by an asterisk. (B) Expression of a PRE-2–GFP fusion protein. Whole cell extracts were prepared from 16-hr liquid SCs and sexually differentiated plate cultures (SCM) of untransformed wild-type (WT) strain 74a and a Δ*pre-2*::*hph* P*ccg-1*::*pre-2-GFP*::*his-3*$^+$ *mat a* strain (P2a17). Samples containing 15 μg of total protein were resolved on 7.5% SDS–PAGE gels and subjected to Western analysis using GFP-specific antiserum as described in *Materials and Methods*. The arrow indicates the full-length PRE-2–GFP fusion protein in submerged cultures of strain P2a17 (∼90 kDa). (C) *In vivo* localization of the PRE-2–GFP protein. The P2a17 strain (Δ*pre-2*::*hph*, P*ccg-1*::*pre-2-GFP*::*his-3*$^+$, *mat a*) was cultivated on 2% water agar plates and observed under a fluorescent compound microscope (top two rows) or a laser-scanning confocal microscope (bottom). The distribution of the PRE-2–GFP protein was analyzed in trichogynes (top right) and conidia (middle right). A protoperithecium expressing the fusion protein was scanned at 5-μm intervals and the 3-dimensional image was reconstructed (bottom right). The left panels are control images viewed in phase contrast for panels on the right side of the figure. Bar, 40 μm.

(Figure 3B). In contrast, Δ*pre-2 mat A* strains underwent normal sexual development (Figure 3A) and exhibited directional growth, coiling, and fusion with *mat a* conidia (Figure 3B). Reintroduction of *pre-2*$^+$ in *trans* into a Δ*pre-2 mat a* strain restored female fertility (Figure 3A) and chemotropic growth of trichogynes toward *mat A* males (Figure 3B). These observations are consistent with the proposed function of *pre-2* as a pheromone receptor specific to *mat a* strains of *N. crassa*. In accordance with previous results (Kim and Borkovich 2004), Δ*pre-1* strains displayed *mat A*-specific defects in female fertility and chemotropic interaction with male cells (Figure 3, A and B). Δ*pre-1* Δ*pre-2* double mutants were female sterile in both mating types (Figure 3, A and B). The double mutants did not produce perithecia (Figure 3A) and their trichogynes failed to grow toward, coil around, or fuse with male cells of opposite mating type (Figure 3B).

Pheromone and pheromone receptor determine the identity of a gamete in N. crassa

In nature, successful mating and sexual development in *N. crassa* require two strains of opposite mating type (*e.g.* wild-type crosses in Figures 3, 4, and 6). In a previous study, we used a genetically engineered *mat a* strain that inappropriately expresses high levels of *ccg-4* (m1a70) (Kim and Borkovich 2006) to demonstrate that pheromones and receptors are initial determinants for sexual identity. Conidia from the m1a70 strain (Δ*mfa-1 ccg-4*^{ec} *mat a*) attracted trichogynes from *mat a* protoperithecia naturally expressing *pre-2* and induced early signs of fertilization, but they ultimately failed to produce ascospores.

In this set of experiments, we explore the requirements for PRE-2 and CCG-4 in strains with swapped identity. We implemented two genetically engineered strains of opposite mating type in these studies: P1C4A46 (Δ*pre-1* Δ*ccg-4 pre-2*^{ec} *mat A*), which inappropriately expresses *pre-2* in the *mat A* background, and m1a70, which produces *ccg-4* transcript in the *mat a* background (see above; Table 1; Kim and Borkovich 2006).

The control cross involving two wild-type strains of opposite mating type resulted in production of perithecia and ascospores (Figure 4, top). In a cross between a wild-type *mat A* female and a m1a70 male, conidia from the m1a70 strain failed to induce perithecial development or chemotropic growth of trichogynes from wild-type *mat A* protoperithecia, despite the presence of different mating types (Figure 4, middle). On the other hand, in a cross of P1C4A46 as a female to m1a70 as a male, trichogynes produced by P1C4A46 displayed normal directional growth toward, coiling around, and fusion with m1a70 conidia; underwent normal perithecial development; and produced viable ascospores (Figure 4, bottom). These results indicate that an attraction signal between male and female cells can be transmitted if a pheromone and a pheromone receptor are compatibly paired, but successful perithecial development and ascospore production requires the presence of both mating type loci.

Coexpression of PRE-2 and CCG-4 in the mat A background induces self-stimulation but does not lead to meiosis and ascospore production

We have shown that the *pre-2* transcript is expressed to high levels in the *mat a* background (Figure 1), while *ccg-4* mRNA is abundant in *mat A* strains (Bobrowicz *et al.* 2002; Kim and Borkovich 2006). The expression profile and deletion

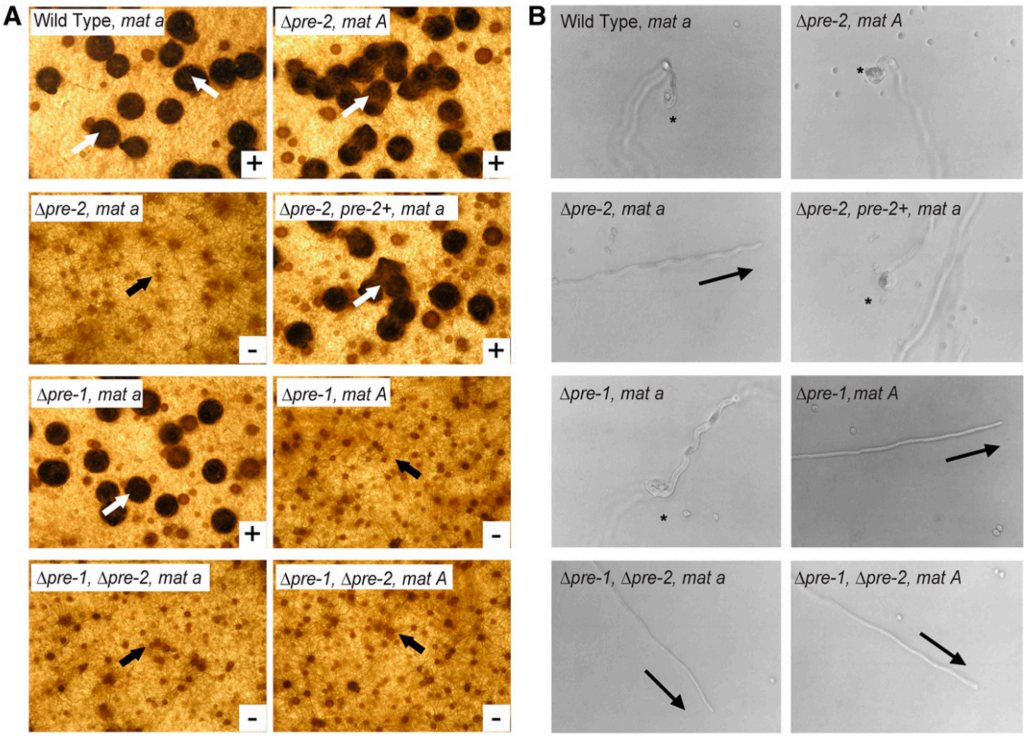

Figure 3 Female fertility assays. (A) Formation of perithecia. Protoperithecia from the indicated strains were fertilized with conidia from wild-type strains of opposite mating type and perithecial development was evaluated after 6 days. White arrows show examples of mature perithecia, while black arrows indicate unfertilized protoperithecia. Strains are wild-type 74a (WT *mat a*), wild type 74A (WT *mat A*), 23a (Δ*pre-2*, *mat a*), 23A (Δ*pre-2*, *mat A*), 16a (Δ*pre-1*, *mat a*), 16A (Δ*pre-1*, *mat A*), P1P2a (Δ*pre-1*, Δ*pre-2*, *mat a*), and P1P2A (Δ*pre-1*, Δ*pre-2*, *mat A*). (B) Trichogyne attraction. Trichogynes from the indicated strains were tested for their ability to grow toward and fuse with microconidia from a wild-type strain of opposite mating type. Asterisks indicate positive chemotropic response and coiling events, while arrows point to the direction of trichogyne growth in cases of nonattraction. Strains are the same as in A.

phenotypes for *pre-2* and *ccg-4* are consistent with a model in which the PRE-2 protein interacts with the CCG-4 peptide to facilitate mate recognition and sexual development in *N. crassa* (Poggeler and Kuck 2001; Kim and Borkovich 2006). One way to test this hypothesis is to determine whether spontaneous sexual development occurs when *pre-2* and *ccg-4* are coexpressed in the same strain. Therefore, we constructed *mat A* strains (expressing *ccg-4*) that also produce *pre-2* mRNA under the control of the mating-type–regulated *pre-1* promoter or the constitutive *ccg-1* promoter. The Δ*pre-1 mat A* background was used to ensure that *pre-2* was the only pheromone receptor expressed in these strains.

When *pre-2* was expressed under the control of the *pre-1* promoter in a Δ*pre-1 mat A* background, trichogynes exhibited self-attraction by adhering to one another and forming loops and bundles (strain P1A35; Figure 5A). Production of higher levels of *pre-2* mRNA using the *ccg-1* promoter in the same background (P1A46; see Figure 6B) resulted in more intense self-stimulation (Figures 5B and 6A). Even vegetative hyphae of strain P1A46 showed signs of self-recognition, including formation of looping hyphae on VM or SCM solid medium (Figure 5B; only SCM culture is shown). When starved for nitrogen, the P1A46 strain initiated protoperithecial development. With continued incubation (and in the absence of male cells of opposite mating type), the protoperithecia darkened and enlarged to the size of 2- to 3-day-old perithecia observed in wild-type crosses (Figure 6A). However, the structures in the P1A46 strains lacked defining features of mature perithecia, such as beaks and ostioles (Figure 6A), and did not develop ascogenous hyphae or ascospores (Figure 6A). Deletion of *ccg-4* or *gna-1* in the P1A46 background (strains P1A46-ccg-4 and P1A46-gna-1; Table 1) blocked enlargement and melanization of protoperithecia (Figure 6A), resulting in a phenotype similar to that of wild type or Δ*pre-1* controls (Figure 6A). These results are consistent with a requirement for the appropriate pheromone and the Gα subunit GNA-1 to initiate perithecial development.

Influence of the opposite mating type gene and/or elimination of meiotic silencing on perithecial development in self-stimulating strains

We next explored whether introduction of the opposite mating-type gene would impact perithecial development in strains expressing a compatible pheromone–receptor pair. Interestingly, ectopic expression of the *mat a* (*mat-a-1*) gene in the P1A46 background (strain P1A46-68; Δ*pre-1 pre-2*^{ec} *mat a*^{ec} *mat A*; Table 1) led to production of slightly larger perithecia than those observed in P1A46 alone, although ascospore development was not observed (Figure 6A). This result suggests that the presence of both mating-type genes may allow further progression of perithecial development, as long as the genetic background contains a compatible pheromone–receptor pair. However, the observation that the *mat a-1* transcript could not be detected in strain P1A46-68, containing an ectopic copy of *mat a-1* (Figure 6B), indicates that this effect is either independent of, or results from, very low levels of *mat-a1* expression.

It has been shown that the *Sad-1* gene, encoding a RNA-dependent RNA polymerase, is required for meiotic silencing

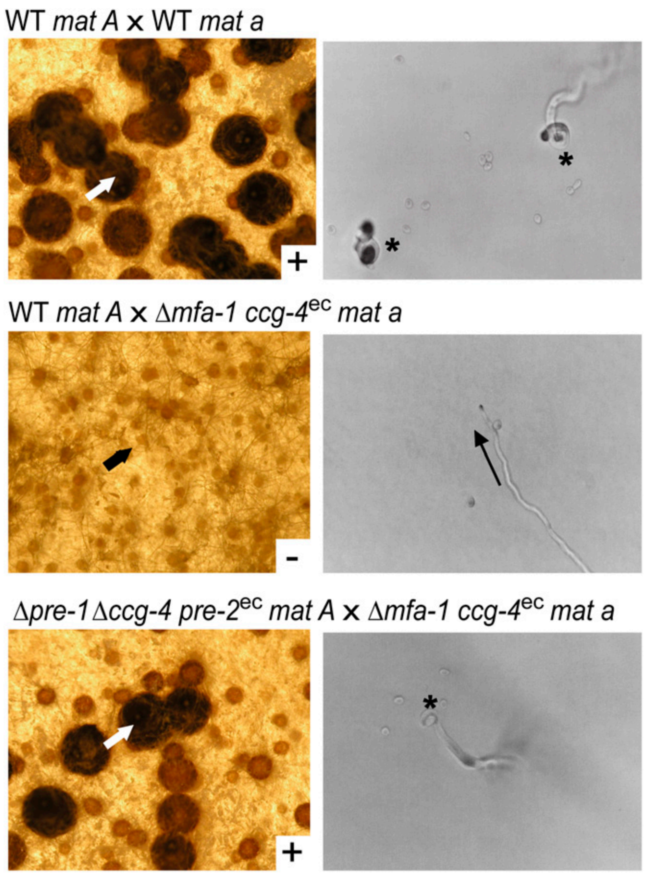

Figure 4 Perithecial development of strains with switched identity due to heterologous expression of *ccg-4* or *pre-2*. Strains used are wild-type (WT) 74A (WT *mat A*), wild-type 74a (WT *mat a*), m1a70 (Δ*mfa-1*, *ccg-4*^{ec}, *mat a*), and P1C4A46 (Δ*pre-1*, Δ*ccg-4*, *pre-2*^{ec}, *mat A*). Note that *ccg-4*^{ec} is P*ccg-1*::*ccg-4*::*his-3*$^{+}$ and *pre-2*^{ec} is P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$. (Left) Perithecial development in crosses between the indicated strains. The first strain listed was cultured as the female, while the second was used as the male. White arrows indicate ascospore-producing normal perithecia (top and bottom), while the black arrow indicates an unfertilized protoperithecium (middle). (Right) Strains used as females in the crosses shown on the left were cultured on 2% water agar plates and trichogynes analyzed for attraction to microconidia from the second strain listed for each cross. Chemotropic responses were monitored and photographed. Asterisks (top and bottom) indicate directional growth, coiling, and fusion events involving trichogynes, while the arrow (middle) indicates the direction of trichogyne growth in cases of nonattraction.

in *N. crassa* (Shiu *et al.* 2001). *Sad-1* silences genes that are in different positions in the genomes of the two parents involved in a sexual cross. Silencing is also observed when an extra copy of a gene is present at an ectopic site in one parent, but not the other. Our self-stimulating strains are not involved in a sexual cross with another partner. However, these strains do contain extra copies of *pre-2* (at the endogenous and *his-3* loci) and/or an ectopic copy of *mat a* in the *mat A* background. We reasoned that the presence of an extra copy of *pre-2* or the ectopic copy of *mat a* might be subject to silencing. Therefore, we examined a possible effect of the *Sad-1* gene on perithecial development and expression of *pre-2*.

Elimination of *Sad-1* in the Δ*pre-1 pre-2*^{ec} *mat A* and Δ*pre-1 pre-2*^{ec} *mat a*^{ec} *mat A* strain backgrounds enhanced perithecial development, with enlargement to the size observed in wild-type heterozygous crosses and formation of beaks and ostioles (Figure 6A). However, ascospores were never formed. To determine whether *pre-2* or *mat a-1* were being silenced at the transcriptional level, we analyzed mRNA amounts using Northern blots (Figure 6B). Compared to the levels observed in the wild-type *mat a* background, the amount of *pre-2* mRNA was greatly elevated in strain Δ*pre-1 pre-2*^{ec} *mat A*, expressing *pre-2* under control of the *ccg-1* promoter. Introduction of *mat a-1* and/or deletion of *Sad-1* did not influence the expression of *pre-2* in the Δ*pre-1 pre-2*^{ec} *mat A* background (Figure 6B). *mat a-1* mRNA could not be detected in any of the *mat A* strains containing ectopic copies of *mat a-1*, indicating that the low level of expression of *mat a-1* is not due to meiotic silencing. These results suggest that while perithecial development is enhanced in the Δ*pre-1 pre-2*^{ec} *mat a*^{ec} Δ*Sad-1 mat A* strain when meiotic silencing is eliminated, this is not due to an effect on the expression of *pre-2* or *mat-a-1*.

Sexual development of heterokaryons requires one compatible receptor–pheromone pair and GNA-1

In the experiments described above, we were unable to achieve high levels of expression of both mating-type genes in the same cell. We reasoned that expression of *mat A* and *mat a* from different nuclei may be necessary for a single cell to complete sexual development in *N. crassa*. In addition, this study and previous work indicate that the PRE-1 receptor signals through the Gα protein GNA-1 (Kim and Borkovich 2004). Therefore, we explored the requirement for receptors, pheromones, and the Gα protein GNA-1 in cells containing nuclei of both mating types. In *N. crassa*, hyphae from two strains can fuse to form a single mycelium (heterokaryon) containing nuclei from the two strains. Heterokaryons can be isolated by mixing strains containing two different auxotrophic markers on minimal medium (forcing conditions; reviewed in Davis and Deserres 1970). The mating-type genes are involved in this process, as efficient fusion (near–wild-type growth rates) is only observed between same mating-type strains (Beadle and Coonradt 1944). However, heterokaryons can be forced between two auxotrophic strains of opposite mating type, with growth rates $\sim$23% of same–mating-type heterokaryons (Beadle and Coonradt 1944). Our analysis included the Δ*pre-1*, Δ*pre-2*, Δ*pre-1* Δ*pre-2*, Δ*gna-1*, and/or *a*^{m1} genotypes and the forcing markers were *his-3* (histidine auxotroph) and *pan-2* (requires pantothenate). All pairs of opposite mating-type strains formed heterokaryons that grew on minimal medium, while control auxotrophic homokaryons did not exhibit any visible growth or colony formation under these conditions.

Heterokaryons between *his-3* and *pan-2* strains (used as controls) formed protoperithecia $\sim$7 days after inoculation onto SCM plates. Many of these protoperithecia grew darker

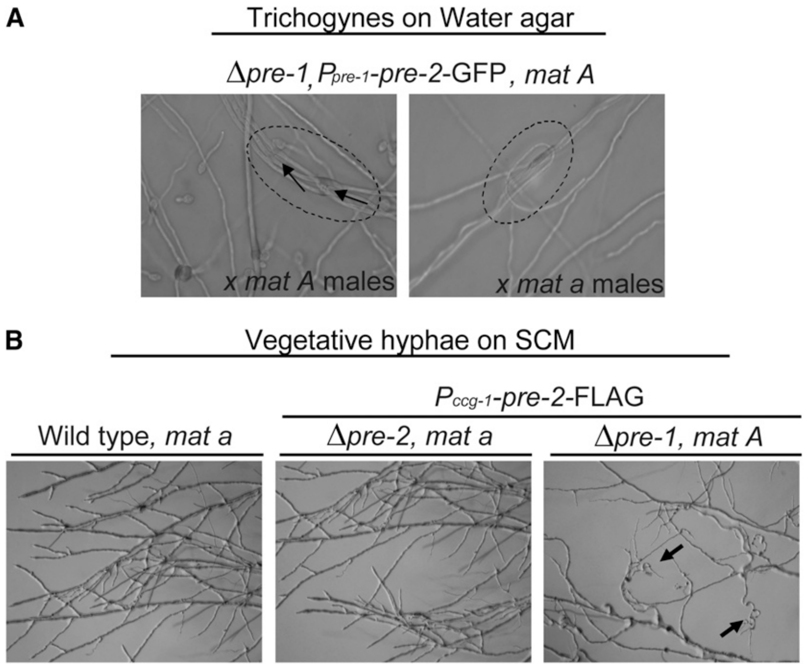

Figure 5 Self-stimulation of trichogynes or vegetative hyphae in Δ*pre-1*, *mat A* strains with coexpression of *pre-2* and *ccg-4*. (A) Self-attraction and adhesion of trichogynes from a strain expressing *pre-2–GFP* under control of the *pre-1* promoter. The P1A35 strain (Δ*pre-1*::*hph*, P*pre-1*::*pre-2-GFP*::*his-3*$^{+}$, *mat A*; abbreviated Δ*pre-1*, *pre-2*–GFP in figure) was cultured on 2% water agar plates and subjected to a trichogyne assay (see *Materials and Methods*) using microconidia from wild-type strain 74A (*mat A*, left) or 74a (*mat a*, right) as the male. While displaying no response to either *mat A* or *mat a* male cells, the trichogynes from P1A35 expressing *pre-2* under the control of the *pre-1* promoter in the *mat A* background (producing normal levels of *ccg-4* pheromone) were attracted to and fused with one other, forming bundles or loops (circled area). The arrows indicate fusion events between trichogynes. (B) Altered growth of vegetative hyphae from a strain expressing *pre-2–FLAG* under control of the *ccg-1* promoter when cultivated under nitrogen starvation conditions. Strain 74A (wild-type *mat A*), P2a46 (Δ*pre-2*::*hph*, P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$, *mat a*), and P1A46 (Δ*pre-1*::*hph*, P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$, *mat A*) strains were grown on SCM plates for 12–20 hr and observed for apical extension of vegetative hyphae. The arrows indicate the curled vegetative hyphae in P1A46 that result from normal expression of the *ccg-4* pheromone coupled with high-level production of the *pre-2* pheromone receptor (under control of the *ccg-1* promoter; see also Figure 6B).

(melanized) and enlarged during days 8–10 and continued development to normal-sized perithecia (Figure 7, top). The perithecia produced and ejected viable ascospores within 5 weeks of inoculation. Heterokaryons formed between Δ*pre-1 mat A* and Δ*pre-1* Δ*pre-2 mat a* or between Δ*pre-2 mat a* and Δ*pre-1* Δ*pre-2 mat A* strains also developed ascospore-producing perithecia, albeit more slowly than controls (Figure 7, top), and the perithecia were fewer in number. The observation of fertility in these heterokaryons is significant, as crosses performed between two homokaryons of the indicated genotypes are sterile. Therefore, the results cannot be explained by heterokaryon breakdown, as the remaining receptor in the Δ*pre-1* or Δ*pre-2* nucleus does not recognize the predicted highly expressed pheromone produced by the other nucleus. These observations are more consistent with a bypass of the pheromone-mediated chemotropic response that leads to cell fusion in these heterokaryons. In heterokaryons produced by two Δ*pre-1* Δ*pre-2* double mutants, protoperithecia darkened without enlargement and perithecial development and ascospore production were not observed (Figure 7, top). This finding indicates that at least one nucleus in the heterokaryon needs to express a pheromone receptor for meiosis and ascospore production to occur.

We next examined the involvement of GNA-1 (Gα) in perithecial development of heterokaryons. As observed for heterokaryons of Δ*pre-1* and Δ*pre-2* single and double mutants, heterokaryons formed between a wild-type and a Δ*gna-1* strain underwent slow and less efficient, but ultimately successful, perithecial development and produced viable ascospores (Figure 7, middle). On the other hand, protoperithecia from heterokaryons of two Δ*gna-1* strains became melanized, but were unable to develop into perithecia or produce ascospores (Figure 7, middle), similar to heterokaryons formed between two Δ*pre-1* Δ*pre-2* strains. These findings indicate that at least one nucleus in the heterokaryon must express *gna-1* to allow completion of sexual development.

The *mat a*^{m1} strain does not express any pheromone or pheromone receptor genes and is both male and female sterile (Figure 1C; Bobrowicz *et al.* 2002; Kim *et al.* 2002; Kim and Borkovich 2004). Heterokaryons formed between *mat a*^{m1} and a wild-type strain occasionally formed barren perithecia (Figure 7, bottom). However, *mat a*^{m1} + Δ*pre-1* or Δ*pre-2* single or double mutant heterokaryons completely lacked perithecia and did not produce ascospores (Figure 7, bottom). These results suggest that each nucleus must contribute a compatible receptor or pheromone for meiosis and ascospore development to occur.

Taken together, our results demonstrate that meiosis and perithecial development can proceed in a heterokaryon-containing nuclei of opposite mating type as long as GNA-1 and one compatible pheromone–receptor pair are expressed, even if the pheromone is produced at low levels. Furthermore, forced fusion of normally sterile strains bypasses the requirement for cell fusion mediated by chemotropism. The data support roles for pheromones, receptors, and GNA-1 in postfertilization events in *N. crassa*.

Discussion

In this study, we have characterized the relationship between the pheromone receptor, PRE-2, and its pheromone

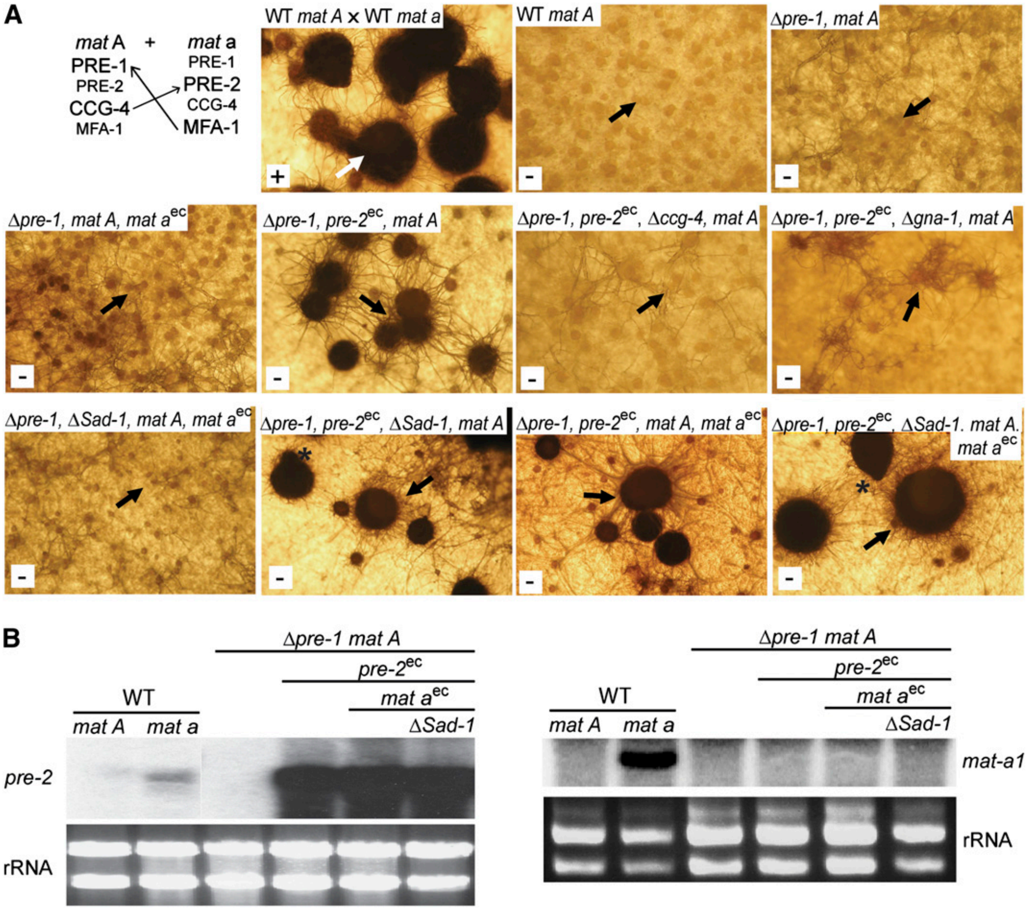

Figure 6 Perithecial development in the absence of opposite mating-type males. (A, top left) Cross of wild-type strains 74A and 74a (positive control for normal perithecial development). Protoperithecia from 6-day-old SCM cultures of strain 74A were fertilized with 74a and resulting perithecia were photographed 4 days later. The white arrow indicates an example of a normal perithecium. For all other panels, SCM plate cultures were grown for 10 days without fertilization. Strains are wild-type 74A (WT *mat A*), 16A (Δ*pre-1*, *mat A*), P1A68 (Δ*pre-1*, *mat A*, *mat a*^{ec}), P1A46 (Δ*pre-1*, *pre-2*^{ec}, *mat A*), P1C4A46 (Δ*pre-1*, Δ*ccg-4*, *pre-2*^{ec}, *mat A*), P1Gα1A46 (Δ*pre-1*, *pre-2*^{ec}, Δ*gna-1*, *mat A*), P1A46-68-Sad1 (Δ*pre-1*, Δ*Sad-1*, *mat a*^{ec}, *mat A*), P1A46-Sad1 (Δ*pre-1*, *pre-2*^{ec}, Δ*Sad-1*, *mat A*), P1A46-68 (Δ*pre-1*, *pre-2*^{ec}, *mat a*^{ec}, *mat A*), and P1A46-68-Sad1 (Δ*pre-1*, *pre-2*^{ec}, Δ*Sad-1*, *mat a*^{ec}, *mat A*). The white box in the lower left corner of each panel indicates whether the strain produced ascospores (+ or −). Black arrows point to examples of protoperithecia or barren perithecia (enlarged bodies) developing in unfertilized cultures. The asterisks indicate beak formation in self-stimulated perithecia from the P1A46-68–Sad1 strain. Note that *pre-2*^{ec} is P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$, while *mat a*^{ec} is *mat a*::*bar*. (B) Expression of *pre-2* and *mat a* in self-stimulating strains. Total RNA was isolated from 6-day-old SCM plate cultures of wild-type *mat A* (74A), wild-type *mat a* (74a), Δ*pre-1*, *mat A* (16A), Δ*pre-1*, *pre-2*^{ec}, *mat A* (P1A46), Δ*pre-1*, *pre-2*^{ec}, *mat A*, *mat a*^{ec} (P1A46-68), and Δ*pre-1*, *pre-2*^{ec}, *mat A*, *mat a*^{ec}, Δ*Sad-1* (P1A46-68-Sad1). Note that *pre-2*^{ec} is P*ccg-1*::*pre-2-FLAG*::*his-3*$^{+}$, while *mat a*^{ec} is *mat a*::*bar*. Samples containing 30 μg of total RNA were used to prepare Northern blots that were probed with *pre-2* (left) and *mat a* (right). rRNA is used as a loading control.

CCG-4. These results, along with previous work, also delineate the basic requirements for receptors, pheromones, mating-type loci, and the Gα protein GNA-1 during attraction and fusion of male and female cells and postfertilization events in *N. crassa*. Our prior work showed that strains lacking the Gα protein GNA-1 are female sterile, with a block in attraction of trichogynes by males and that loss of pheromone causes male sterility. In an earlier study, we also showed that coexpression of a pheromone–receptor pair leads to perithecial development in homokaryons (Kim and Borkovich 2006). Here, we show that while a strain inappropriately expressing the opposite mating-type pheromone receptor is self-attracting and produces perithecia, deletion of either the pheromone gene or *gna-1* blocks this attraction. We demonstrate a possible role for meiotic silencing in self-stimulation, as introduction of the Δ*Sad-1* mutation (Shiu *et al.* 2001) leads to production of beaks and ostioles in these perithecia. However, in no case are ascospores produced, suggesting that the presence of opposite mating-type nuclei is required for meiosis.

Using forced heterokaryons containing nuclei of both mating types, we demonstrate that meiosis and ascospore production depend on the presence of one compatible receptor–pheromone pair and the *gna-1* gene. These requirements do not involve a pheromone response between two cells, as some of the compatible combinations contain strains that are sterile in crosses as homokaryons. Instead, they point to roles for receptors, pheromones, and GNA-1 in postfertilization events. Our results are relevant to a related *Neurospora* species with a pseudohomothallic mode of sexual reproduction, *N. tetrasperma*. During sexual development, *N. tetrasperma* predominantly produces ascospores containing two nuclei, each of a different mating type (Raju 1992a). Heterokaryons containing these two nuclei are self-fertile and do not require an outside mating partner for sexual reproduction. This situation is very similar to the fertility we observed in forced heterokaryons between opposite mating type strains of *N. crassa*.

A complete replacement of the *mat A* gene region with *mat a* has been accomplished in *N. crassa*, with production of a fertile strain that behaves as a *mat a* parent in sexual crosses (Chang and Staben 1994). Likewise, gene replacement of *mat a* with *mat A* leads to fertility and ascospore

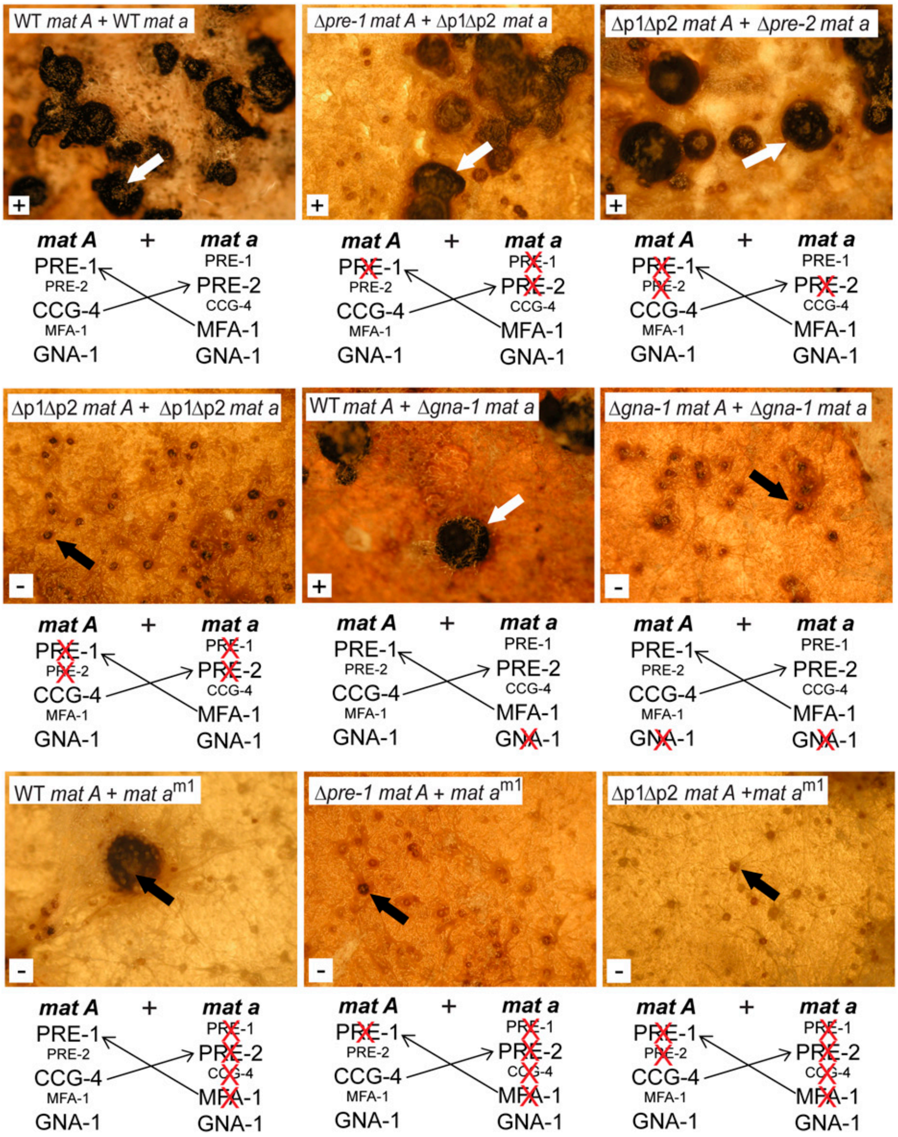

Figure 7 Sexual development of heterokaryons formed between opposite mating-type strains. Conidial suspensions of two opposite mating-type strains with different auxotrophic markers (*pan-2* and *his-3*) were mixed and used to inoculate the center of SCM plates lacking pantothenate and histidine. The plates were incubated to facilitate fusion of hyphae from the two strains and formation of a heterokaryon that can grow in the absence of pantothenate or histidine. All *mat A* strains are in the *his-3* background, while all of the *mat a* strains (with the exception of *mat* a^{m1}) are *pan-2* auxotrophs. The *mat* a^{m1} strain carries the *ad-3B* allele and is an adenine auxotroph. Perithecial development was monitored and photographs were taken 45 days after inoculation. White arrows indicate mature perithecia that produced viable ascospores, while black arrows point to melanized protoperithecia or enlarged but barren perithecia. The white box in the lower left-hand corner of each photo indicates whether the heterokaryon produced ascospores (+ or −). WT indicates wild-type alleles of the *pre-1*, *pre-2*, *gna-1*, and *mat a* genes. Δp1Δp2 = Δ*pre-1*, Δ*pre-2*. Strains (and relevant genotypes) are FGSC 6103 (WT *mat A*), pan-2 a (WT *mat a*), 16Ahis3 (Δ*pre-1*, *mat A*), 23apan2 (Δ*pre-2*, *mat a*), P1P2h3A (Δ*pre-1*, Δ*pre-2*, *mat* A), P1P2h3a (Δ*pre-1*, Δ*pre-2*, *mat a*), Δ1his-3 (Δ*gna-1*, *mat A*), Δ1pan-2 (Δ*gna-1*, *mat a*), and a^{m1}.

production (Glass *et al.* 1988). In contrast, ectopic integration of the *mat A* gene in a genome containing a defective *mat a* allele (a^{m1} mutant) led to production of perithecia, but no ascospores (Glass *et al.* 1988). Similarly, ectopic introduction into *N. crassa* of opposite mating-type genes from *Podospora anserina* that are homologs of *N. crassa mat A* and *mat a* leads to self-mating and perithecial enlargement, but ascospores are not produced (Arnaise *et al.* 1993). These last two findings are consistent with our results testing the effect of an ectopic copy of *mat a* in the self-stimulated strains. We observed that sexual development is blocked at a stage prior to meiosis, suggesting a requirement for the mating-type genes to be expressed by two different nuclei for completion of sexual development in *N. crassa*. Our ability to force perithecial development by heterologous expression of a compatible receptor–pheromone pair suggests that the perithecial development observed in the earlier studies in strains containing two mating-type genes may have resulted from elevated expression of the cognate receptor or pheromone. This hypothesis is consistent with our observation that introduction of the *mat a* gene did not result in a large increase in the size of the barren perithecia produced in the self-stimulated strains in our study.

The results of our attempts to engineer *N. crassa* to homothallism can be compared to the situation in the true homothallic *Neurospora* species *N. africana* and *N. terricola*. Hybridization experiments indicate that *N. terricola* contains both a *mat A* and *mat a* gene in the haploid genome (Glass *et al.* 1988). In contrast, *N. africana* does not contain a *mat a* gene, but possesses *mat A-1*, encoding a protein that is 93% identical to, and can substitute functionally for, the *N. crassa mat A-1* gene (Glass and Smith 1994). In contrast to our results with *N. crassa*, these findings indicate that sexual fertility can be observed in *Neurospora* species containing one or both mating-type genes in the same nucleus. In the future, it would be interesting to investigate expression of pheromones, receptors, and other sexual development genes in *N. terricola* and *N. africana*, to identify regulatory proteins that are expressed in *N. africana* and/or *N. terricola*, but not in *N. crassa*. The results of such experiments may shed light on the evolution of homo- and heterothallism in *Neurospora*.

Requirements for pheromone receptors and mating-type genes have also been explored in the homothallic self-fertile fungus *Aspergillus nidulans*. *A. nidulans* contains two different mating-type genes in its genome, on two different chromosomes, and both are essential for self-fertility and sexual spore production (Paoletti *et al.* 2007). Self-fertility also requires the presence of the GprA and GprB (PRE-2 and PRE-1 homologs, respectively) pheromone receptors; deletion of either receptor gene significantly reduces fruiting body production and viable sexual spores, and loss of both receptors abolishes homothallic reproduction (Seo *et al.* 2004). Thus, the requirement for receptors for sexual fertility and meiosis is shared between *N. crassa* and *A. nidulans*. However, in contrast to our results with *N. crassa*, the two mating-type genes can function at different positions in the genome to regulate self-fertility and meiosis in *A. nidulans*.

In this and an earlier study, we observed that mutation of a pheromone receptor gene in the mating type with low expression level leads to reduced production of the other normally highly expressed receptor. Here, we further show that the effect of the receptor mutations also influences expression of both pheromone precursor genes. These results suggest that the low amount of receptor mRNA produced is physiologically relevant and may influence expression of the other normally abundant receptor and the pheromones. However, the low expression of the non–mating-type favored receptor may result from cross-talk and is not sufficient to allow mating as a female. Furthermore, the reduction in the highly expressed receptor does not interfere with sexual fertility. In fact, we only observe a function for the low-expressed pheromone in forced heterokaryons where cell–cell communication has been bypassed, suggesting that low levels of receptor may be adequate for postfertilization events such as nuclear fusion.

Taken together, our data indicate that receptors and pheromones function in postfertilization events in *N. crassa*. Such a scenario has been observed in basidiomycete mushroom fungi, with these components needed subsequent to fertilization, for nuclear migration in the dikaryon and clamp cell formation (Casselton and Feldbrugge 2010). In yeast, experiments exploring fusion of spheroplasts from same mating-type cells showed that nuclear fusion requires prior exposure to mating pheromone (Rose *et al.* 1986). Elucidation of the mechanism by which receptors and pheromones regulate postfertilization events is an area ripe for future investigation and one that will yield much information about the extent to which self- and non–self-discrimination modulates sexual reproduction in ascomycete fungi.

Acknowledgments

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