RESEARCH ARTICLE

Neurospora crassa fmf-1 encodes the homologue of the *Schizosaccharomyces pombe* Ste11p regulator of sexual development

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Abstract

The *Neurospora crassa fmf-1* mutation exerts an unusual 'perithecium-dominant' developmental arrest; $fmf-1 \times fmf-1^+$ cross becomes arrested in perithecial development regardless of whether the mutant participates in the cross as the male or female parent. We localized fmf-1 to the LG IL genome segment between the centromere-proximal breakpoint of the chromosome segment duplication Dp(IL)39311 and the centromere. By mapping crossovers with respect to RFLP markers in this region we further localized fmf-1 to an approximately 34-kb-genome segment. Partial sequencing of this segment revealed a point mutation in the gene *NCU 09387.1*, a homologue of the *Schizosaccharomyces pombe stel11*⁺ regulator of sexual development. The fmf-1 mutation did not complement a *NCU 09387.1* deletion mutation, and transformation with wild-type *NCU 09387.1* complemented fmf-1. *S. pombe* Stel11 protein (Stel1p) is a transcription factor required for sexual differentiation and for the expression of genes required for mating pheromone signalling in *matP* and *matM* cells. If FMF-1 also plays a corresponding role in mating pheromone signalling in *Neurospora*, then protoperithecia in an $fmf-1 \times fmf-1^+$ cross would be unable to either send or receive sexual differentiation signals and thus become arrested in development.

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Introduction

The *female and male fertility-1 (fmf-1)* mutant of *Neurospora* crassa has an astonishing phenotype; its cross with the wild-type (*fmf-1* × *fmf-1*⁺) becomes arrested in sexual development when the perithecia attain only 40% of their normal diameter, regardless of whether the mutant participates in the cross as the male or female parent (Johnson 1979). That is, *fmf-1* exerts an unusual 'perithecium-dominant' developmental arrest. Intriguingly, the mutant is heterokaryon-recessive; *fmf-1* × *fmf-1*⁺ crosses are fertile if the *fmf-1* mutant nucleus is derived from an [*fmf-1* + *fmf-1*⁺] heterokaryon. Using the *tolerant (tol)* mutation to suppress mating type heterokaryon incompatibility (Newmeyer 1970), Johnson (1979) constructed the heterokaryon [(*tol pan-1 fmf-1 A*) + (*tol trp-4 fmf-1*⁺a)], crossed it with multiply marked *mat a* strains, and mapped *fmf-1* to between *mat* and *cr-1* on

linkage group (LG) I; tentatively, close to *arg-1*. Sequencing of the *N*. *crassa* genome revealed that *mat* and *cr-1* are separated by > 3.3 Mbp (Galagan *et al.* 2003).

The objective of this study is to map the *fmf-1* mutation to a single gene, in the expectation that identification of the *fmf-1* gene would help us to formulate hypotheses to account for its unique mutant phenotype. This long-mysterious (or, long-neglected) gene has now been found to be the homologue of *Schizosaccharomyces pombe ste11*⁺. The Ste11 protein (Ste11p) is a transcription factor that regulates sexual development and is required for mating pheromone signalling in both *matP* and *matM* cells. Homology between *fmf-1*⁺ and *ste11*⁺ suggests that the FMF-1 protein might play a corresponding role in mating pheromone signalling in *Neurospora*. If that be the case, the *fmf-1*×*fmf-1*⁺ perithecial development could be arrested because the protoperithecia are unable to either send or receive mating pheromone signals.

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We dedicate this paper to our friend Namboori Bhaskara Raju, Stanford University, to recognize his many quiet, yet very significant, contributions to fungal biology. He continues to be such a fine example for the rest of us.

Keywords. perithecial development; chromosome segment duplications; mating pheromones; yeast; *Neurospora*.

Materials and methods

Gene, phenotype and protein symbols

Gene symbols are italicized, while phenotype symbols are not. Phenotypes are designated with caps (e.g., Hyg+ or HygR, and Fmf+ and Fmf-) and no gene number is used. Protein names are all caps and no italics (i.e., FMF-1).

Neurospora crassa strains

Neurospora genetic analysis was essentially as described by Davis and De Serres (1970). Unless otherwise indicated, all the N. crassa strains used were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas, USA. They included the standard Oak Ridge strains 74-OR23-1 A (FGSC 987) and OR8-1 a (FGSC 988), the auxotrophic mutant strains arg-3 a (FGSC 1069) and his-1 A (FGSC 401), and the *fmf-1* mutant strains *fmf-1*; *pyr-3* A (FGSC 3108) and fmf-1; tol pan-1 A (FGSC 3109). The pyr-3 and pan-1 mutations are unlinked to fmf-1 and confer auxotrophy for uracil and pantothenate (Perkins et al. 2001). The tol (tolerant) mutation suppresses mating type heterokaryon incompatibility (Newmeyer 1970), and was used to recover Dp(IL) 39311 strains (see below) which contain both mat A and mat a idiomorphs. The semi-dominant Sad-1 (Suppressor of ascus dominance-1) mutation suppresses meiotic silencing and, thereby, enhances the productivity of $Dp \times eu$ ploid crosses (Shiu et al. 2001). The Sad-1 A (FGSC 8740) and Sad-1 a (FGSC 8741) strains were kindly provided by the late Robert L. Metzenberg.

The helper-1 strain (FGSC 4564; a^{m1} ad-3B cyh-1) contains *fmf-1*⁺, and the inactive a^{m1} allele of *mat a* that does not trigger mating type heterokaryon incompatibility but makes it a passive partner when the heterokaryon is used as a parent in a cross. Only the nuclei of the active partner participate in karyogamy and the production of sexual progeny (Perkins et al. 2001). The ad-3B mutation confers adenine auxotrophy. Heterokaryons between the *fmf-1* mutant strains and the helper-1 strain were isolated by selection for complementation of the auxotrophic markers, and they will henceforth be referred to as [fmf-1; pyr-3 A] and [fmf-1; tol pan-1 A], respectively. An arg-3 fmf-1 a segregant was obtained from the cross arg-3 $a \times [fmf-1; pyr-3A]$ (out of 315 segregants examined, including 150 arg⁻) and used to make the heterokaryon with helper-1. This heterokaryon will be referred as [arg-3 fmf-1 a].

The translocation strains T(IR;VR;IR > VII)In(VL;VR)AR173 a (FGSC 2469), T(IR > VII)P7442 mo a (FGSC 3209) and T(IL > IIR)39311 a (FGSC 1246) are described by Perkins (1997) and will be referred to as T(AR173)a, T(P7442) a and T(39311) a, respectively. The wildisolated (W) mat A strains Chemax (P4212) Colonia Paraiso (P4212), Mauriceville-1c (FGSC 2225) and Mughalsarai (P0736) were used for RFLP mapping.

Generation of RIP-induced his-2 mutants

5' AAGCTGGTGCCAAT-The oligonucleotides GTTCTTGC 3' and 5' AGGCAGATCACCGTTCAAATCG 3' were custom purchased from Bioserve (Hyderabad, India) and used as primers to amplify a 1463 bp segment of the his-2 gene by PCR, and the amplified DNA was cloned into the EcoRV site of the plasmid vector pCSN44 (Staben et al. 1989). The resulting plasmid (pSIhis2) was transformed by electroporation into conidia of the his-3 A strain (FGSC# 6103), and transformants were selected on hygromycin-medium. The PCR reaction conditions, other molecular methods, and transformation protocols were as described by Bhat et al. (2004). The primary transformants were crossed with OR a, and among their progeny we obtained several that were hygromycin-resistant, prototrophic and *mat a*, consistent with the hypothesis that the transgene, designated Dp(his-2), was unlinked to his-3 (and mat). The Dp(his-2) a strains were crossed with fmf-1; pyr-3A and with the wild-isolated mat A strains Colonia Paraiso (P4212) and Mauriceville-1c (FGSC 2225). Dp(his-2) A progeny from these crosses were then crossed with OR a to target RIP to the *his*- 2^+ locus on the LG I derived from the *fmf*-1 or the wild-isolate parent (Galagan and Selker 2004). Eight RIP-induced his-2 alleles were obtained on LG I's bearing fmf-1; the fmf-1 his-2 A segregant #45 was used in the crosses that established that *fmf-1* is not distal to *his-2* (see Results). Three and 21 his-2 mutants, respectively, were obtained on the LG I's derived from the Colonia Paraiso and Mauriceville-1c strains, and they were used in the localization of *fmf-1* via RFLP mapping.

Inverse PCR

Genomic DNA $(2 \mu g)$ of the translocation strain was digested with the restriction enzyme in a $20 \mu l$ reaction for 4–5 h. One μg of the digested DNA $(10 \mu l)$ was ligated using 12 Weiss units NEB ligase for 16 h at 16°C in a 500 μl reaction volume. The circularized DNA was then ethanol precipitated, resuspended in TE and 0.1 μg was used as template for PCR using primers INVF 5' AGCACGTGGGAGAGAATCCTT 3' and INVR2 5' TGGATCTGGAAGGTGTCACCT 3'.

Molecular markers to localize fmf-1

PCR based RFLPs were identified to distinguish between the genomes of OR and the wild-isolated strains. The oligonucleotide primers and restriction enzymes used to obtained these RFLPs are listed in tables 1 and 2 of electronic supplementary material at http://www.ias.ac.in/jgenet/.

Results

fmf-1 localizes to between the proximal breakpoints of Dp(IL) 39311 and Dp(IR) AR173

The chromosome segment duplications Dp(IL) 39311, Dp(IR) AR173 and Dp(IR) P7442 partition the mat - cr-1

interval into five segments (I-V; figure 1). To determine whether *fmf-1* is covered by any of these *Dp*'s, we crossed the translocation strains T(AR173) a and T(P7442) a with the heterokaryon [fmf-1; pyr-3 A] and the T(39311) a translocation strain with the heterokaryon [fmf-1; tol pan-1 A] (see Materials and Methods for strain descriptions). Among the progeny from crosses of these translocations with normal sequence strains, a subset is duplicated for the translocated segment (i.e., *Dp*(*AR173*), *Dp*(*P7442*) and *Dp*(*39311*)), and the duplication progeny (Dp) can be identified in a subsequent cross by the barren phenotype characteristic of $Dp \times$ euploid crosses (Perkins 1997). Barren crosses make normal looking perithecia, but produce exceptionally few ascospores. Barrenness is caused, at least in part, by a presumably RNAi-based process called meiotic silencing by unpaired DNA, that silences duplication-borne genes, including those required for the completion of meiosis and ascus development (Shiu et al. 2001). The semi-dominant Sad-1 mutation suppresses meiotic silencing and, consequently, $Dp \times$ Sad-1 crosses show increased productivity (Shiu et al. 2001). Thus Dp progeny are barren in crosses with OR but relatively more fertile in crosses with Sad-1.

Sixty-three f₁ progeny from $T(AR173) a \times [fmf-1; pyr-3]$ A] were crossed with OR strains of opposite mating type and 27 were phenotypically Fmf⁺ a (i.e. fertile in crosses with OR A), whereas 36 were Fmf⁻ A (i.e. arrested in crosses with OR a). Of the Fmf⁻ A strains, 28 were Ura⁻ and could be used to select heterokaryons with the *helper-1* strain (see Materials and methods). Four heterokaryons were barren in crosses with OR a but more productive with *Sad-1 a*, suggesting that their non-*helper* component was genotypically Dp(AR173), and since this component as a homokaryon had the Fmf⁻ mutant phenotype, it followed that Dp(AR173) does not cover *fmf-1*. The remaining 24 heterokaryons tested gave fertile crosses with OR *a*; therefore, their non-*helper* component must be of the non-duplication *fmf-1 A* genotype.

Of 34 f₁ segregants examined from [*fmf-1; pyr-3 A*] \times T(P7442) a, 11 were Fmf⁺ a and 23 were Fmf⁻ A. Of the latter, 14 were Ura⁻ and used to make heterokaryons with helper-1 and crossed with OR a and Sad-1 a. One [(Fmf⁻; $Ura^{-} A$) + *helper-1*] heterokaryon was barren in the cross with OR a, but fertile with Sad-1 a, thus indicating that its non-helper component was Dp(P7442) in genotype, and allowing us to conclude that Dp(P7442) does not cover fmf-1. Of 38 f₁ segregants examined from [*fmf-1*; tol pan-1 A] \times T(39311) a, 19 were Fmf⁺ a, eight were Fmf⁻ A and 11 had the 'square' morphology characteristic of strains duplicated for *mat* and whose mating type incompatibility is suppressed by tol. By PCR we confirmed that the 11 strains contained both mat idiomorphs (data not shown), as would be expected for strains with the Dp(39311), tol genotype. These strains were phenotypically Fmf⁻; therefore, we could conclude that Dp(39311) does not cover fmf-1.

Since *fmf-1* was not covered by any of the three duplications, the mutation must lie either in interval II or IV shown in figure 1. The *arg-3* and *his-2* loci are, respectively, the



Figure 1. Genetic localization of *fmf-1*. (A) Dp(IL) 39311, Dp(IR) AR173 and Dp(IR) P7442 partition the *mat - cr-1* interval into the segments I–V. (B) The proximal breakpoint of Dp(39311) (i.e., $T(39311)^R$), and the distal breakpoint of Dp(AR173) (i.e., $T(AR173)^R$), were localized onto contigs 7.6 and 7.72 (dotted red lines, see text for details). Inverse PCR from $T(AR173)^R$ and sequencing revealed that the proximal breakpoint of Dp(AR173) is in a centromeric repeat sequence (*Cen-1*) (dotted and dashed red line). Since *fmf-1* is not covered by the *Dp*'s, nor is it distal to *his-2* (see text), it must lie in II, i.e., proximal to $T(AR173)^R$. (C) Crossovers that produced prototrophic progeny from *arg-3 fmf-1 a* × *fmf-1⁺ his-2 A* were mapped with respect to the RFLP markers 7.3, *A8*, *R3L3-1* and *R3L5* on contigs 7.6 and 7.82 and this localized *fmf-1* to the 34281 bp segment (plus a gap) bounded by *A8* and *R3L3-1*. The *fmf-1* strain displayed a T to A mutation in the *NCU 09387.1* gene within this region.

proximal-most auxotrophic markers on LG IL and IR, and they are covered, respectively, by Dp(IL) 39311 and Dp(IR)AR173. We used repeat-induced point mutation (RIP) to introduce a his-2 mutation into the fmf-1 background (see Materials and methods), and the resulting (fmf-1 his-2) A strain was made into a heterokaryon with helper-1 and crossed with arg-3 a. If fmf-1 is distal to his-2, then all the prototrophic crossover progeny from this cross should be Fmf⁺. Of 62 prototrophic segregants examined, seven were Fmf-A, 53 Fmf⁺ A and two Fmf⁺ a. Since 11% of the progeny were Fmf⁻ in phenotype, it followed that *fmf-1* is not distal to his-2 and therefore it must map to interval II shown in figure 1, i.e. between the proximal breakpoints of Dp(IL)39311 and Dp(IR) AR173, a region operationally defined to be pericentromeric on LG I (Rosa et al. 1997). The two Fmf⁺a segregants must represent double crossovers with one crossover between mat and arg-3 and the other between arg-3 and *fmf-1*.

Dp(39311) and *Dp*(*AR*173) proximal breakpoints localized onto the genome sequence

The arg-3 - his-2 genome segment is partially sequenced (Galagan et al. 2003); arg-3 is on contig 7.6, 305.4 kbp from the contig's proximal end, whereas his-2 is on contig 7.72, ~74.4 kbp from the proximal end. Four other contigs; 7.82 (~107.5 kbp), 7.62 (~181 kbp), 7.92 (69.4 kbp) and 7.86 (~95.6 kbp) are located between contigs 7.6 and 7.72. Thus, the arg-3 and his-2 interval contains 833.3 kbp of sequence and five gaps of unknown lengths (figure 1). To localize the proximal breakpoints of Dp(IL) 39311 and Dp(IR) AR173 onto this sequence, we obtained a T(39311), tol pan-1 a segregant from [fmf-1; tol pan-1 A] \times T(39311) a, and then crossed it and the T(AR173) a translocation (T) strain with the wild-isolated (W) mat A strains Mughalsarai and Chemax. The Dp(IL) 39311 progeny were identified by their 'square' morphology and the Dp(IR) AR173 A progeny by the barrenness of their crosses with OR a. Dp segregants from a $T \times W$ cross contain both the T and W alleles of any RFLPs that are within the translocated segment but, barring crossovers, only the W allele for RFLPs outside this segment. By testing a series of linked RFLP markers for coverage by the Dp, we identified covered and uncovered markers bracketing the Dp breakpoint, and then progressively narrowed down the 'breakpoint interval' by iterating this process with additional RFLPs from within the interval (data not shown; see Vyas et al. 2006 for a figure describing the method). The Dp(IL) 39311 proximal breakpoint was further localized to between a pair of oligonucleotides that failed to PCR amplify a product when DNA from the T strain was used as template but could amplify a ~ 1.5 kbp fragment from OR (and W) DNA, thus suggesting that the Dp(IL) 39311 proximal breakpoint lay within this amplicon. This amplicon shared ~200 bp overlap with a more distal amplicon which was amplified from both OR and T DNA. Therefore the breakpoint was in the 1345 bp interval bounded by the proximal primers of the two amplicons, the 5' bases of which were, respectively, nucleotides 38240 and 36895 on the '+' strand of contig 7.6.

The Dp(IR) AR173 strains were duplicated for all RFLP markers examined on contigs 7.72, 7.86 and 7.92, but not for any of the markers tested on contigs 7.62 and 7.82 (data not shown). In parallel, the distal breakpoint of Dp(IR) AR173was localized to a 102 bp interval bounded by two oligonucleotide primers (INV-R1 and INV-R2) whose 5' bases were at positions 41534 and 41432 on the '-' strand of contig 7.72 (data not shown). Then, using T(AR173) a genomic DNA as template and primers INV-R2 and INV-F that anneal to sequences immediately distal to the Dp(IR) AR173 distal breakpoint, we performed an inverse PCR to 'jump' into the sequence immediately proximal to the proximal breakpoint. The breakpoint-proximal sequence (accession number EU815636; see figure 1 in electronic supplementary material) was almost identical to a repeat sequence found in the centromeric regions of LG VII and LG III, which suggested that the proximal breakpoint of Dp(IR) AR173 was in a copy of this repeat in the LG I centromeric/pericentromeric region. That the T(IR) AR173 breakpoint is in putative Cen-1 sequences is consistent with inferences from earlier studies that this breakpoint is the most proximal landmark on LG IR (Rosa et al. 1997) and implied that contigs 7.86 and 7.92 represent LG IR sequences proximal to 7.72, whereas contigs 7.62 and 7.82 were probably sequences from LG IL (figure 1). This suggested that the *fmf-1* candidate region was now wholly in LG IL, in the genome segment proximal to the proximal breakpoint of Dp(IL) 39311. This candidate region contains \sim 327 kbp of sequence and three gaps (figure 1).

Further localization of fmf-1 and identification of a candidate gene

We targeted RIP to the his-2 locus in LG I of the wild-isolated strains Colonia Paraiso and Mauriceville-1c (see Methods) so that the resulting his-2 A strains retained the wild-isolate genetic background in the LG I pericentromeric region. These strains were then crossed with the heterokaryon (arg-3 fmf-1 a), thereby creating crosses that were heterozygous for fmf-1, as well as for RFLPs between OR and Colonia Paraiso (CP) or Mauriceville-1c (M) in the *fmf-1* candidate region (figure 2). The progeny from these crosses will henceforth be referred to, respectively, as CP or M type. Most of the progeny were auxotrophic for either arginine or histidine, but one could easily select prototrophs resulting from rare crossovers between arg-3 and his-2 (figure 2). The prototrophs were phenotypically Fmf⁻ or Fmf⁺ depending on whether the crossover occurred between arg-3 and fmf-1 (region i; figure 2) or between *fmf-1* and *his-2* (region ii; figure 2). Of the 58 prototrophs examined, 43 were Fmf^- (26 CP + 17 M) and 15 were Fmf^+ (5 CP + 10 M). Each crossover was mapped with respect to the RFLP markers 7.3, A8, R3L3-1 and R3L5 on contigs 7.6 and 7.82 (figure 1). Among the prototrophs, the OR versus wild-isolate allele fraction should increase going from markers on the left (7.3) to those on the



Figure 2. Prototrophic progeny produced in the cross $arg-3 fmf-1 a \times fmf-1^+ his-2 A$. (A) Crossovers in region i produce progeny with the fmf-1 phenotype, whereas crossovers in region ii produce progeny with the $fmf-1^+$ phenotype. The blue line represents the OR genetic background and the brown line the genetic background of the wild-isolate (Colonia Paraiso or Mauriceville-1c). (B) The 'right-most' region i crossover was between the Colonia Paraiso allele of A8 and the OR allele of R3L3-1 and suggested that fmf-1 was to the right of A8 (top panel). The 'left-most' region ii crossover occurred between the Mauriceville-1c allele of A8 and the OR allele of R3L3-1 and suggested that fmf-1 was to the left of R3L3-1 (lower panel). Thus fmf-1 was localized between A8 and R3L3-1.

right (*R3L5*). The fraction of prototrophic Fmf⁻ progeny with the OR allele was 35/43 at 7.3 (22 CP + 13 M), 42/43 at A8 (25 CP + 17 M), and 43/43 at *R3L3-1* and *R3L5*. Thus, the 'right-most' region i crossover was between the Colonia Paraiso allele of A8 and the OR allele of *R3L3-1* (figure 1); therefore *fmf-1* must map to the right of A8. The fraction of prototrophic Fmf⁺ progeny with the OR allele was 0/15 at 7.3 and A8, 1/15 at *R3L3-1* (M) and 2/15 at *R3L5* (1 CP + 1M). That is, the 'left-most' region ii crossover lay between the Mauriceville-1c allele of A8 and the OR allele of *R3L3-1*; therefore *fmf-1* must map to the left of *R3L3-1*. The A8-R3L3-1 interval (i.e., the new candidate region) contained ~34.2 kbp of sequence and one (potentially treacherous) gap (figure 1).

Hardly had we sequenced ~8 kbp of this candidate region in the mutant strain, than we found a T to A transversion mutation that altered the 3' acceptor signal of a putative intron of the *NCU 09387.1* gene in contig 7.82. The wild type *NCU 09387.1* allele has the sequence TATAG-C (the '-' denotes the splice site), which agrees well with the *Neurospora* splice acceptor consensus (Bruchez *et al.* 1993), whereas in the *fmf-1* mutant this signal was TAAAG-C (accession number EU624416). This result identified *NCU 09387.1* as a candidate for *fmf-1*⁺, and suggested that the mutant phenotype was due to a splicing defect.

Evidence that NCU 09387.1 is fmf-1

An NCU 09387.1KO a strain (NCU 09387.1 knockout; FGSC 13541), generated in the Neurospora Genome Project, is available from the Fungal Genetics Stock Center. We found that perithecial development was blocked in crosses of this strain with OR A (data not shown), just as in the *fmf-1* × OR crosses. Conjecturing that the knockout nuclei might be able to participate in a cross if they are derived from a heterokaryon with helper-1, we crossed his-1 A mycelia with NCU 09387.1KO a mycelia to which we had added a suspension of *helper-1* conidia. This 'triparental' cross was fertile, presumably because formation of the heterokaryon [*NCU 09387.1KO a + helper-1*] fostered successful mating between the *NCU 09387.1KO a* and *his-1 A* nuclei. From such mating we obtained ten *NCU 09387.1KO*; *his-1 a* progeny and used them to construct [(*NCU 09387.1KO*; *his-1 a*) + (*arg-3 fmf-1 a*)] heterokaryons. Crosses of all ten heterokaryons with OR A were blocked in perithecial development. Therefore *NCU 09387.1KO* and *fmf-1* did not appear to complement in the heterokaryons, consistent with the idea that both *NCU 09387.1KO* and *fmf-1* affect the same gene.

In other experiments we had found that $[helper-1 + fmf-1 A] \times [helper-1 + fmf-1 a]$ crosses also were blocked in perithecial development (data not shown), which suggested that FMF-1 protein has an ascus-autonomous function that cannot be fulfilled by FMF-1 protein from the parental mycelia. We constructed 10 [*NCU 09387.1KO*; *his-1 a* + *helper-1*] heterokaryons and crossed each with [*helper-1* + *fmf-1 pyr-3 A*]. Again, all ten crosses were blocked in perithecial development, thus demonstrating that *NCU 09387.1KO* and *fmf-1* also do not complement for the presumptive ascusautonomous function. Once again, noncomplementation between *NCU 09387.1KO* and *fmf-1* was consistent with the idea that *NCU 09387.1KO* and *fmf-1* mutate the same gene.

We used the oligonucleotide primers fmf-F 5' CGGGTTTGACCAAGAACAG 3' and fmf-R 5' CCTGTC-CTATCCTCGTTCCA 3' to PCR amplify a 3557 bp segment of wild-type DNA that included the *NCU09387.1* ORF together with sequences 947 bp upstream and 228 bp downstream of the predicted start and stop codons and cloned it into the *Eco*RV site of the vector pCSN44. We transformed the resulting plasmid (*pSI9387*) by electroporation into conidia of strain *arg-3 fmf-1a* and selected several transformants on hygromycin medium. We performed crosses of 32 primary transformants with the strains *ORA* and *Sad-1 A*. Ordinarily, crosses of *arg-3 fmf-1a* with *ORA* and *Sad-1 A*. are sterile, but at least ten transformants yielded ascospores in crosses with both *ORA* and *Sad-1 A*, thereby demonstrating that *pSI9387* contains sequences that can complement the *fmf-1* mutation. This established that NCU 09387.1 and *fmf-1*⁺ are identical.

Discussion

This work has established that *fmf-1* is *NCU09387.1*. The genome sequence facilitated the identification of PCR based RFLPs between the OR and wild-isolate strains and by using RIP to target *his-2* mutations in the wild-isolate backgrounds we could retain RFLP heterozygosity in the *arg-3 fmf-1* × *fmf-1⁺ his-2* crosses and thus localize the *fmf-1* mutation to a genome segment small enough to be easily sequenced in the mutant. Also, the *helper-1* strain and the *Sad-1* mutation made it easier to test for coverage of *fmf-1* by chromosome segment duplications. Tools such as the genome sequence, RIP, *helper-1* and *Sad-1* have become available in *Neurospora* only since 1979.

Interestingly, in the mapping studies to establish that *fmf-1* is not distal to *his-2*, we had obtained 53 single crossovers in the interval arg-3 - fmf-1 and only seven in the interval *fmf-1 - cen 1 - his-2*, despite the fact that the former interval was only 317 kbp in size and had only one gap, whereas the latter was 513.4 kbp and had four gaps. That is, fewer crossovers occurred per kbp in the putatively larger interval than in the putatively smaller one, possibly because the larger interval includes the centromere and therefore might be more heterochromatinized.

NCU 09387.1 is an orthologue of the Schizosaccharomyces pombe stell gene (Borkovich et al. 2004). Therefore it was likely that *fmf-1* and *stel1* share similar functions, and that the unusual *fmf-1* mutant phenotype might be explainable in terms of the properties uncovered for its fission yeast homologue. Stell protein (Stellp) is a highly unstable HMG-box transcription factor that is induced by starvation and, in turn, activates a number of genes required for sexual differentiation (Sugimoto et al. 1991; Mata and Bahler 2006). Stel1p also stimulates the transcription of its own gene, thus setting up a positive feedback loop (Kunitomo et al. 2000). During vegetative growth Stel1p is localized primarily in the cytoplasm (Qin et al. 2003) and its DNA-binding activity is inhibited by Cdk-phosphorylation (Kjærulff et al. 2007); this presumably safeguards against inappropriate activation of the sexual developmental pathway. As cells starve, dephosphorylation of Ste11p makes it available for nuclear import and leads to increased expression of genes required for mating pheromone signalling and sexual differentiation of both matP and matM cells (Qin et al. 2003). If FMF-1 plays a corresponding role in mating pheromone signalling in *Neurospora*, then it is likely to be required both for expression in protoperithecial trichogynes of receptors for mating pheromones secreted by the conidia, as well as in the conidia for the synthesis and secretion of the pheromone. Conidial trichogyne attractants and their protoperithecial receptors are mating type-specific (Bistis 1983; Kim and Borkovich 2004, 2006). Therefore, in an *fmf-1* × *fmf-1*⁺ cross, *fmf-1* protoperithecia would fail to express receptors for the pheromone of the *fmf-1*⁺ conidia, and *fmf-1* conidia would fail to express the pheromone to signal to the *fmf-1*⁺ trichogynes. Consequently, neither the *fmf-1*⁺ nor the *fmf-1* protoperithecia are fertilized, and perithecial development is arrested at the same stage in both cases, regardless of whether the mutant strain participates in the cross as the male or female parent.

Johnson (1979) had reported that the $[(fmf-1^+; tol A) + (fmf-1; tol a)]$ heterokaryon was 'self-fertile' whereas the [(fmf-1; tol A) + (fmf-1; tol a)] heterokaryon was 'self-sterile'. That is, if mating type heterokaryon incompatibility is overcome by use of tol, then the FMF-1 protein from a single $fmf-1^+$ allele can satisfy the requirement of this protein for sexual differentiation of 'fmf-1' protoperithecia and conidia and fertilization is not hampered. In other words, the unusual 'perithecium-dominant' and 'heterokaryon-recessive' fmf-1 mutant phenotype emerges from the fact that mating type heterokaryon incompatibility prevents the diffusion of FMF-1 protein between the parental mat A and mat a mycelia.

The [fmf-1 + helper-1] heterokaryon bears some analogy to mixed cultures of fmf-1 and N. intermedia, N. sitophila, N. tetrasperma or N. discreta strains of the same mating type; each component of the mixed culture is sterile in its cross with OR and, presumably, if any heterokaryon were to form, it would not be subjected to mating type heterokaryon incompatibility. However, in contrast to the ready productivity of [fmf-1 + helper-1] × OR crosses, attempts to 'cross' the mixed cultures with OR remained nonproductive (SVI and DPK, unpublished results). This provided experimental evidence against the occurrence of interspecies heterokaryons, possibly because other kinds of vegetative heterokaryon incompatibilities also can prevent diffusion of the FMF-1 protein.

In summary, this work has identified *fmf-1* as the *Neurospora* homologue of *S. pombe stell*⁺. The Stellp is known from other studies to be required for mating pheromone signalling in *S. pombe matP* and *matM* cells. Our results suggest that the FMF-1 protein might play a corresponding role in mating pheromone signalling in *Neurospora*. This hypothesis enables us now to envision how perithecial development might be arrested in an *fmf-1* × *fmf-1*⁺ cross.

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