Molecular and Functional Analyses of poi-2, a Novel Gene Highly Expressed in Sexual and Perithecial Tissues of *Neurospora crassa*

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The *poi*-2 gene is highly and specifically expressed in starved and sexual tissues of the filamentous fungus *Neurospora crassa*. It encodes a 27-kDa protein, as shown by in vitro transcription and translation. The POI2 protein contains a hydrophobic signal sequence at the amino terminus followed by novel 16 tandem repeats of 13 to 14 amino acid residues; all repeats are separated by Kex2 processing sites. Repeat-induced point mutation (RIP)-mediated gene disruption was used to generate *poi*-2 mutants, and the mutated sequences showed either one of two distinct patterns: typical RIPs (GC-to-ACT transitions) or insertion-deletion (indel) mutations. Although the *poi*-2 strains contained numerous mutations, all retained intact open reading frames (ORFs) of various lengths. They showed greatly reduced vegetative growth and protoperithecial formation and low viability of their sexual progeny. All *poi*-2 mutants had similar defects in male fertility and the mating response, but the nature of female fertility defects varied and corresponded to the length of the residual *poi*-2 ORF. Mutants with ORFs of approximately normal length occasionally completed sexual development and produced viable ascospores, while a mutant with a severely truncated ORF was female sterile due to its inability to form protoperithecia. Thus, *poi*-2 is essential for differentiation of female reproductive structures and perithecial development as well as for normal vegetative growth. The POI2 protein is involved in the mating response, probably as a component in the pathway rather than as a pheromone.

*Neurospora crassa* is a heterothallic fungus with two mating types (mat*a* and mat*A); the decision whether to undergo asexual or sexual development is made based on environmental signals. Under conditions of nitrogen starvation, light, and low temperature (35, 38), vegetative hyphae undergo differentiation in preparation for the sexual reproductive pathway, aggregating to form a spherical prefruiting body, the protoperitheium. When fertilized with a nucleus of the opposite mating type, the protoperitheium develops into a peritheium (fruiting body), within which ascospores are formed from successive developing hook-shaped hyphae (croziers). Inside the developing ascus, the diploid zygote undergoes meiosis followed by a postmeiotic mitosis. The resulting eight nuclei are sequestered into eight linearly ordered homokaryotic, haploid ascospores, and the ascospores are ejected after further maturation.

*N. crassa* has a long history in classical and biochemical genetics, but relatively little is known about sexual development at the molecular level. Nelson and Metzenberg isolated 14 genes that are preferentially expressed during sexual development and named them *svd* genes, for sexual development (31). A mutant with a disrupted *svd-10* gene, which was renamed *ascus development 1* (*asd-l*), was isolated using the repeat-induced point mutation (RIP)-based reverse genetic technique (43, 44). Another mutant (*asd-2*) was created by random integration of transforming DNA into a gene required for sexual development. In later studies, additional *ascus development* genes, *asd-3* (30) and *asd-4* (12), were identified, and mutations in these genes were generated by the RIP process. All four *asd* genes are essential for sexual development. More recently, genes encoding pheromones and pheromone receptors have been identified using reverse genetic analysis of expressed cDNAs or genes (2, 19, 20). To date, two types of fungal pheromone precursor genes have been identified; despite variations in their amino acid sequences and lengths, their critical structures are conserved among the fungi. The budding yeast *MFA*-like pheromone genes encode long precursor molecules that undergo multistep maturation processes (8) and produce peptides with a C-terminal CAAX motif (C, cysteine; A, aliphatic; and X, any amino acid residue). In contrast, the *MFC*-like genes encode polypeptides containing multiple repeats of a putative pheromone sequence bordered by Kex2-like protease processing sites, with a hydrophobic signal sequence at the amino terminus. In previous work, the novel *poi*-2 (plenty of it) gene was identified as an expressed sequence tag and shown to be expressed at high levels specifically in starved tissues (29). Based on its pattern of expression, the *poi*-2 gene was predicted to function in sexual development. In this study, the *poi*-2 gene was analyzed at the molecular and functional levels. The results showed that *poi*-2 is an essential gene and that its product is required during both vegetative growth and sexual development of *Neurospora*.

**MATERIALS AND METHODS**

*Neurospora* strains and plasmids. The following *N. crassa* strains were used in this study (a refers to mat*,* and A refers to mat*): the wild-type 74A (74-OR23-IV A [FGSC 24890]) and ORS a (FGSC 2490) strains and the mutant strains fl A (FGSC 4317), Fl A (FGSC 4347), arg-10 mat A (FGSC 7217), qa-2 arg-9 inl al-2 a (RLM 63-01), A*mm* (un-3 ad-3A nic-cyb-1 A*mp* [FGSC 4570]), A*mp* (ad-3B cyb-1 A*mp* [FGSC 4564]), jnf-1 jnr-3 A (FGSC 3108), and the a tol/A tol heterokaryon (pan-1 nic-3 al-1 tol A + pan-1 tol al a) (RLM 58-12 + RLM 58-12).
A homothallic mat A idiomorph Neurospora species, *N. africana* (FGSC 1740), was also used. FGSC indicates the Fungal Genetics Stock Center, University of Missouri, Kansas City, Mo., and RLM indicates a laboratory strain of *R. L. Metzenberg*, FGSC strains 4411, 4416, and 4450 through 4487 were also used to carry out restriction fragment length polymorphism (RFLP) mapping. The specialized plasmid pMSN1 (31), which contains the *qa-2* gene of *N. crassa*, was used as a selectable marker for transformants potentially receiving the *poi-2* cDNA. The pGEM3zf (+) vector (Promega, Madison, WI) was used in subcloning of *poi-2* genomic DNA from the pMOCosC N. crassa genomic library (33). The genomic clone of *poi-2* was obtained by subcloning an approximatly 6-kb PsI fragment from the G2F5 cosmid genomic clone.

**Media and culture conditions.** *N. crassa* cultures were maintained in liquid Vogel's minimal medium (VM medium) (47) with 1.5% sucrose and required supplements. For vegetative growth, strains were cultured either in liquid VM medium or on solid VM medium in petri dishes (100 mm) or in race tubes (50 cm in length and 18 mm in diameter). All crosses were carried out on synthetic crossing medium containing 1% sucrose and required supplements (49). For growth of cultures for RNA or protein isolation, conidia were inoculated as described previously (29, 31). For vegetative conditions, cells were inoculated in liquid VM medium at a final concentration of 10⁶ cells/ml and were cultured in the dark at 30°C with agitation for 5, 14, or 24 h. For mating conditions, cells were inoculated in liquid synthetic crossing medium at a final concentration of 10⁶ cells/ml and incubated without agitation at 25°C in the light for 3 to 6 days. For preparation of the peritrichal RNAs and proteins, the *flb* strain *fla* or *fla fla* was grown on crossing plates covered with sterile Miracloth circles (Calbiochem) and fertilized with a heavy conidial suspension of the opposite mating type, 74A or ORS-a, respectively. Either 7 or 9 days after fertilization, the perithecia were scraped from the crossing plates, immediately frozen, and ground in liquid nitrogen using a mortar and pestle.

**DNA sequencing and sequence analyses.** The full cDNA and genomic *poi-2* sequences were determined using the dideoxy chain termination method (41) using the Applied Biosystems (ABI) PRISM dye terminator kit (Perkin-Elmer) and ABI model 377 DNA sequencer. The genomic clone of *poi-2* was obtained by subcloning an approximately 6-kb PsI fragment from a *N. crassa* genomic library (33). To identify potential homologs, the DNA sequence of the *poi-2* sequence to score the occurrence of RIP and identify indels.

**Cell-free syntheses of *poi-2* cDNA.** A 5'-cap analog, m<sup>G</sup>cap(G<sup>5</sup>ppp(G<sup>5</sup>)), was added to the reaction mixtures to improve the translatable quality of the synthetic mRNA. The resulting capped and polyadenylated mRNA encoding POI2 was then used for in vitro translation reactions using a Neurospora cell-free translation system (48). A luciferase cDNA encoding a 61-kDa protein was used as control for both experiments and prepared in the same manner. The [35S]methionine-labeled translation products were separated on sodium dodecyl sulfate-polyacrylamide gels and visualized by autoradiography.

**Western blot analysis.** Total proteins were extracted from the wild-type strain using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) by following the manufacturer's instructions. The proteins were separated on 16.5% Tricine-sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) by electroblotting. Blots were blocked with 2% skim milk in TBS (30 mM Tris-HCl, pH 7.4, 130 mM NaCl) for 2 h at room temperature and incubated with purified anti-POI2 antibodies (diluted 1:5,000) in TBS with 2% skim milk and 1% aprotinin at 4°C overnight. Blots were washed three times for 10 min in TBS with 0.1% Tween 20 and incubated with anti-rabbit goat immunoglobulin G (H+L) conjugated with horseradish peroxidase (from Pierce). After three 10-min washes in TBS with 0.1% Tween 20, blots were visualized using the SuperSignal West Pico chemiluminescence detection kit (Pierce) as instructed by the manufacturer.

**RFLP mapping of the *poi-2* gene.** RFLP mapping was performed to determine the localization of a common restriction site in *poi-2* and to predict whether *poi-2* corresponded to any genes that had been previously identified by mutation using classical genetic techniques. This method uses RFLPs as genetic markers and examines the ordered progeny from a cross of a multiply marked laboratory strain (multicent-2, *a*, in an Oak Ridge genetic background) with a wild-collected strain (Mauriceville-1c A) (28). For this analysis, the cosmid encoding *poi-2* was linearized with a restriction enzyme, labeled, and hybridized with Southern blots of digested genomic DNA from mapping strains.

**Gene disruption experiments (RIP).** The pMSN1 plasmid and *poi-2* cDNA were cotransformed into freshly harvested conidia of the *qa-2 aro-9 inl al-2* (RLM 63-01) strain via electroporation (6). The *qa-2* transformants were selected based on their ability to grow without the aromatic amino acid supplement, which is needed for growth of untransformed *qa-2 aro-9* double-mutant strains. Single-colony isolates of the primary transformants were defined as diploids if they failed to produce conidia containing diploid genomic DNA (90). Transformants containing only two copies of *poi-2* (an endogenous *poi-2* and one extra copy introduced by transformation) were identified by Southern analysis. Transformants with two copies of the *poi-2* gene were then crossed with the *arg-10 m A* strain (FGSC 7217), which has two linked auxotrophic mutations (*arg-10* and *mt*) mapping near the *poi-2* gene and contains no duplicated *poi-2* sequences. The progeny of these crosses were plated onto medium lacking the arginine and nicotinamide required by the auxotrophic mutations. Progeny were selected by INC, in an Oak Ridge genetic background) with a wild-collected strain (Mauriceville-1c A) (28). For this analysis, the cosmid encoding *poi-2* was linearized with a restriction enzyme, labeled, and hybridized with Southern blots of digested genomic DNA from mapping strains.

**Sequence analysis of the *poi-2* gene of selected meiotic progeny.** The sequences of 20 potentially disrupted *poi-2* genes were examined. A 2.0-kb fragment of the endogenous *poi-2* gene was amplified from progeny genomic DNA by PCR. Approximately 0.5 µg of genomic DNA was amplified with primers 1 and 2 (5'-TATGTTGATGCGCCAGTGAG-3' and 5'-GGGTTGTGTGTCCTGGTCC-3'), respectively. The PCR product was verified by agarose gel electrophoresis, purified using the QIAquick Spin PCR purification kit (QIAGEN), and sequenced using nested primers 3, 4, and 5 (5'-GGAGTAGAGGTACCTGG-3', 5'-AGTCAGACCTCTAAGCCAC-3', and 5'-CTCTGATCGACGTTTCTCCG-3', respectively) with the ABI PRISM dye terminator kit (Perkin-Elmer) and ABI model 377 DNA sequencer. Primers 1 to 4 were designed from the flanking sequences of the *poi-2* gene, and primer 5 was designed from the *poi-2* cDNA sequence. The *poi-2* sequences were compared with the *poi-2*-2 sequence to score the occurrence of RIP and identify indels.

**Phenotypic analyses.** (i) Linear growth rate tests. Growth rates of the *poi-2* mutant strains were tested on minimal, complex, and low-nitrogen media at 25°C, 30°C, or 37°C, using race tubes (7).

(ii) Fertility tests. To analyze the role of the *poi-2* gene in sexual development, *poi-2* mutants were crossed to the wild type as either the female (protoperticular) or male (fertilizing) parent to detect dominant mating-specific defects (31). When used as a male, a small drop of conidial suspension was spotted onto *flb* (*fla fla*) strains grown on plates with crossing medium. The *flb* mutants are highly fertile and unable to produce macroconidia (24). These crosses also served to identify the mating type of the mutants, as perithecia formed on either the *flb* on *fla fla* plates. When used as females, the mutants were grown in crossing slants and fertilized with wild-type conidia of the opposite mating type. The *poi-2*
mutant strains were crossed with sibling strains to detect recessive mutations affecting sexual development (31).

(iii) Mating response tests. Attraction between trichogynes of one mating type and conidia of the opposite mating type was assayed to investigate pheromone function in the poi-2 mutants, as described previously (20). An agar strip covered by dense growth of \textit{fla} or \textit{flA} was placed next to a 2% water agar strip, and then a heavy wild-type or poi-2 conidial suspension was streaked onto the 2% water agar approximately 5 mm from the \textit{fluffy} strain. Mating responses were monitored to determine if the distant conidia could stimulate the directed growth of trichogynes and to localize the sites of perithecial production.

RESULTS

Representation of the poi-2 cDNA in various cDNA libraries. The poi-2 gene was identified by the \textit{Neurospora} Genome Project at the University of New Mexico as the second most highly expressed gene under starved conditions (29). In the project, three cDNA libraries were initially constructed using mRNA isolated from conidial (germinating asexual spores), starved mycelial (branching hyphae), and perithecial (fruiting body) tissues. An additional cDNA library, the Westergaard library, was subsequently constructed to represent unfertilized sexual (protoperithecial) tissue (9). More recently, two “time-of-day-specific” cDNA libraries (morning and evening libraries) were constructed and analyzed to investigate the expression of genes in mycelial tissues of \textit{N. crassa} over the course of the circadian day (51). Analysis of these unsubtracted libraries showed that poi-2 clones accounted for 4.2% of the starved mycelial library and 2.4% of the perithecial library, both of which represent starved conditions. No poi-2 cDNAs were identified in the conidial, Westergaard, morning, or evening library.

Sequence analyses. The sequences of selected poi-2 cDNA inserts and the subcloned genomic poi-2 DNA were determined. The genomic poi-2 sequence and the derived amino acid sequence of the predicted POI2 protein are shown in Fig. 1. The gene contains a single intron of 67 nucleotides and an open reading frame (ORF) with good \textit{N. crassa} bias (3, 37) that is preceded by an untranslated stretch of about 80 nucleotides (as predicted by the lengths of the longest poi-2 cDNAs).

The poi-2 ORF encodes a 238-amino-acid-long polypeptide with a predicted molecular weight of 27 kDa, which was supported by in vitro transcription/translation experiments (Fig. 2). The putative POI2 protein contains a predicted transmembrane helix and a signal sequence for secretion at the N terminus (residues 3 to 22 and cleavage between residues 18 and 19, respectively). Also, there are 16 tandem repeats of 13 to 14 amino acid residues starting at residue 25, most of which are flanked by a typical processing site for Kex2-like protease (13) (Fig. 1 and 3A). These repeat sequences contain a novel motif (Fig. 3B), and hydropathy prediction showed a regularly alter-
nating pattern of hydrophilic and hydrophobic regions in the
POI2 protein (Fig. 3C).

To identify potential homologs of poi-2, the DNA and deduced
protein sequences were compared with the nucleotide and pro-
tein databases available through the National Center for Biotech-
nology Information (http://www.ncbi.nlm.nih.gov/BLAST) as well as
with fungal genome databases accessible through the Broad In-
stitute (http://www.broad.mit.edu/annotation/fungi/). The results

FIG. 3. Structural features of POI2. (A) Alignment of 16 tandem repeats. Asterisks indicate residues present in at least 15 of the repeats.
(B) Frequency of all residues in the 16 tandem repeats; integers indicate the number of times the respective residues were present. Alternative
residues at a given site are shown in parentheses, and the histidine and asparagine residues present only in the longer repeats are in square brackets.
Below, a potential consensus sequence of the repeated motif is shown (boxed). (C) Comparison of the hydropathy of POI2 (N. crassa; top) and
Rep1 (U. maydis; bottom). Hydrophobicity patterns were produced by the ProtScale program (http://www.expasy.ch/tools/) using parameters
defined previously by Kyte and Doolittle (23). Window sizes of 5 and 11 were used for POI2 and Rep1, respectively.
of these sequence analyses showed no sequence identity to any genes or proteins characterized to date. However, the POI2 protein showed striking structural resemblance to the Rep1 protein of Ustilago maydis, i.e., a signal for secretion at the N terminus followed by multiple tandem repeats (12 repeats of a 37-amino-acid consensus) showing similar hydropathy (Fig. 3C) (50). Rep1 is an abundant filament-specific cell wall protein that is required for aerial growth of filaments and surface hydrophobicity in the corn smut fungus (50). A tandem repeat structure is also present in budding yeast mating factor (Mfα) precursor (22). Mfα1 and Mfα2 pheromone precursor genes contain four and two tandem repeats, respectively.

Presence of poi-2 homologs in other ascomycetes. Heterologous hybridizations of poi-2 with genomic fungal DNAs confirmed that there is a single copy of the poi-2 gene in the N. crassa genome and no apparent paralogs (Fig. 4), which was supported by BLAST searches against the N. crassa genome database at the Broad Institute (http://www.broad.mit.edu/annotation/fungi/neurospora/). The heterothallic species N. intermedia and N. sitophila contain single copies of a poi-2-related gene, as do the pseudohomothallic species N. tetrasperma and G. tetrasperma. Also, the homothallic species G. reticulosa, N. africana, N. galapagosensis, N. terricola, S. fimicola, S. macrospora, and A. sublineolata contain an apparent poi-2 gene.

Mapping of poi-2. To determine whether poi-2 represents a newly identified gene or one previously characterized, the chromosomal location of poi-2 was determined using the RFLP method (28, 32). The poi-2 gene was mapped to linkage group VII, near cat-2 and cox-8 (approximately 3% and 10% recombination, respectively). That region does not include any genes previously identified as playing a role in sexual development (36).

Expression of the poi-2 gene. The poi-2 cDNA was the second most abundant clone in starved mycelial and perithecial cDNA libraries of N. crassa (as discussed above). Northern analysis was carried out to examine the expression of poi-2 under additional conditions. A forced heterokaryon containing both the mat a and mat A loci was used to mimic mating conditions (31). Also, protoperithecia form efficiently only on the surface, so that only the stationary cultures (floating mycelial mats) grown on crossing medium were expected to contain significant levels of transcripts specific to sexual development.

Northern analysis showed that poi-2 was expressed at high levels in mating conditions and during perithecial development (Fig. 5A, lane 3, and B, lane 5). The expression was high in the forced heterokaryon grown as a floating mycelial mat on crossing medium (Fig. 5A, lane 3) and was low but detectable in the
The poi-2 gene of Neurospora crassa

The poi-2 gene is involved in sexual development and is expressed in both male and female tissues. Two independent transformants were generated using the RIP approach, and these transformants were crossed with a strain containing auxotrophic mutations near the poi-2 gene. The progeny of the crosses were plated onto medium lacking both arginine and nicotinic acid to enrich for progeny derived from the transformed strain, with potential disruption of the endogenous poi-2 gene. About 5% of germinated ascospores displayed slow and sparse hyphal extension.

Based on their extremely poor vegetative growth, 20 potential poi-2 mutants were selected from the transformant I RIP cross, and their poi-2 alleles were examined for the presence of typical RIP mutations (GC-to-AT transitions). Five mutated poi-2 alleles were identified (corresponding to less than 1% of the total progeny from the RIP cross) and designated 1 to 5. These five poi-2 alleles showed an unexpected pattern of mutations; instead of the GC-to-AT transition mutations created by the RIP process, indels (insertion-deletion mutations) were obtained. Surprisingly, the five poi-2 alleles were identical, even though they contained multiple sequence alterations, including an insertion of 81 nucleotides, two deletions of 39 and 81 nucleotides, respectively, as well as 20 point mutations flanking the indels and 2 point mutations within the insertion.

In Table 1, the sequence analysis of the poi-2 mutant alleles is presented. The alignments of the poi-2 protein sequences of the wild type and mutants are shown in Fig. 6.

FIG. 6. Comparison of POI2 protein sequences of the wild type (WT) and mutants. The alignment was obtained using the ClustalW 1.8 Multiple Sequence Alignment algorithm available at BCM Search Launcher (http://dot.imgen.bcm.tmc.edu); default parameters were used. The three typical RIP mutants are designated by their allele numbers, and the eight mutants of identical sequence are indicated as Indel. The entire wild-type protein sequence is shown, and the changes in the poi-2 mutants are noted below. Dots indicate amino acids identical to the wild type, while dashes show the extents of the insertion and deletions.
recognizable after digestion with the AluI restriction endonuclease (not shown).

Eighty potential poi-2 mutants were selected from the progeny of the transformant II RIP cross, again based on their poor vegetative growth. Since a distinctive RFLP pattern of poi-2 indels was established in the initial RIP cross, and digestion with the restriction enzyme AluI could be used to identify some of the typical RIP-generated transition mutations, the poi-2 RFLP patterns of the transformant II progeny were examined (not shown). Of the 80 selected progeny, 6 had RFLP patterns different from that of the wild-type control (corresponding to a frequency of less than 1% of the total progeny). These six poi-2 alleles, designated 101 to 106, were sequenced and further analyzed. They showed one of two distinct patterns of mutations, indicated as indel or RIP; none contained frameshift mutations. The properties of these alleles are summarized in Table 1.

The 103, 105, and 106 poi-2 alleles contained insertion-deletion mutations with multiple sequence alterations. Strikingly, these new indel alleles were identical to the five poi-2 alleles isolated from the initial RIP cross (alleles 1 to 5 [see above]). To determine if the indel mutations were present before the RIP cross, the RFLP patterns of the parental strains were examined (not shown). No evidence of the RFLP pattern associated with the poi-2 indels was obtained, but it is possible that the indels were present in a minority of the parental nuclei.

The other three alleles from the second RIP cross contained typical RIP-generated GC-to-AT transitions, ranging from 37 to 81 nucleotide changes. Alleles 101 and 102 had both G→A and C→T transitions on the sense strand, suggesting that multiple rounds of RIP had occurred, while allele 104 had only C→T transitions on the sense strand, suggesting a single cycle of RIP (5). Alleles 101 and 104 maintained the full-length ORF, while the 102 allele lost the initiation codon, yet it gained a new one near the 3' end of the gene, resulting in an ORF of 48 amino acid residues (Fig. 6 and Table 2). The repeat structures of all mutant alleles are compared in Table 2.

![FIG. 7. Western blot analysis of POI2. (A) Size marker. (B to E) Crude protein extracts prepared from unfertilized and fertilized sexual tissues. Proteins in lanes B, C, and D were prepared from ORS a grown without agitation on crossing medium for 4, 5, and 6 days, respectively. Proteins in lane E were prepared from 7-day-old perithecia (isolated from a cross of fla and 74A). (F) In vitro-translated POI2. The synthetic protein was prepared as described for in vitro transcription and translation (Materials and Methods), except that [35S]methionine was replaced with unlabeled methionine. As shown in Fig. 2, no signal was detected in the control lacking RNA template.](image-url)
Western blot analysis. Western blot analysis was performed to characterize the POI2 polyclonal antibodies raised to a repeat unit of the POI2 protein and to detect the POI2 protein in wild-type and mutant strains. While the preimmune serum did not show any cross-reactivity (not shown), the POI2 antibody recognized a single band in protein extracts from both unfertilized and fertilized sexual tissues (Fig. 7, lanes C to E). Although the poi-2 transcript was abundant in starved mycelial tissue, no POI2 was detected in starved mycelial protein extracts (not shown). When detected in sexual tissues, the apparent size of POI2 (~30 kDa) was fairly consistent with its expected size (27 kDa), suggesting that POI2 may be unmodified and unprocessed in those tissues. The presence of posttranslational processing was not detected in the culture types used in this study. However, recently, evidence for posttranslational processing of POI2 has been detected in VM plate cultures (S. Phillips, H. Kim, and K. Borkovich, personal communication). Such processing might explain the failure to detect POI2 in starved mycelia.

Vegetative growth. To characterize the vegetative phenotypes of poi-2 mutants, linear growth rate was tested on minimal and complex media at 25°C, 30°C, and 37°C. All poi-2 mutants showed very slow and sparse growth on minimal medium as well as poor conidiation. When compared with the wild type, poi-2 mutants exhibited an at least three times slower linear growth rate at 25°C or 30°C, while growth was slightly improved at 37°C (Fig. 8). When supplemented with 0.5% yeast extract, which supports the growth of most Neurospora auxotrophs (7), the poi-2 growth defect was not reversed; thus, poi-2 mutants are not auxotrophic. However, poi-2 mutants gradually reverted to wild-type vegetative growth after prolonged maintenance in culture and subculturing. The poi-2 sequences of revertants were identical to the sequences in the poorly growing original isolates, which suggested that the strains showing good vegetative growth were pseudorevertants. Also, sexual development in the apparent pseudorevertants was similar to that of the wild type. Given the very poor vegetative growth of poi-2 mutants, strong selective pressure would be expected, enriching for revertants with stronger vegetative growth.

When reverted poi-2 (102) A was crossed to a wild-type female, progeny with the original phenotypes of poi-2 (102) could be retrieved from the cross in addition to those with wild-type growth. Approximately 40% of the germinated progeny displayed poi-2 phenotypes, while 60% grew like the wild type. After prolonged incubation at room temperature, about one-third of apparent poi-2 strains displayed pseudoreversion; once a portion of the sparse mycelium on the surface started to form aerial hyphae, heavy conidiation followed within 24 h, and cultures resumed wild-type growth patterns in 2 to 3 days (not shown).

Sexual development and the mating response. The fertility of poi-2 mutants was tested under low-nitrogen conditions. In poi-2 strains with nearly full-length ORFs, protoperithecial development was greatly delayed, and reduced numbers were produced compared to those of the wild type. When crossed with wild-type strains, as either male or female, these poi-2 mutants formed perithecia after a delay, and the perithecia developed slowly and produced a small number of viable ascospores. Spore maturation after ejection was slow as well. When examined a week after spore ejection, the percent of inviable, nonpigmented ascospores was somewhat higher (~10%) than that of a wild-type cross (less than 5%); also, the majority (~80%) of pigmented spores were brown rather than black, suggesting delayed ascospore maturation. Germination efficiency of the recently ejected pigmented ascospores produced in poi-2 by wild-type crosses was no higher than 15%, while the corresponding efficiency from wild-type crosses was 95 to 99%. However, the lightly pigmented ascospores gradually darkened, and 1 to 2 months after ejection, their germination efficiency increased to about 40%.

In homozygous crosses of poi-2 mutants, perithecial development was more greatly delayed than in heterozygous crosses, but small numbers of viable ascospores were eventually produced. However, in poi-2 (102) A, the mutant with a severely truncated ORF, protoperithecial development was absent, re-
DISCUSSION

Based on its high and specific levels of expression in starved tissues of N. crassa, the poi-2 gene was predicted to play a significant role in sexual development (29). Phenotypic analysis of poi-2 mutants has indicated that poi-2 has multiple roles throughout the life cycle of N. crassa. The poi-2 gene was required for (i) hyphal growth and conidiation in vegetative development, (ii) differentiation of female reproductive structures, (iii) the mating response, and (iv) perithecial development and ascospore maturation.

The primary function of poi-2 may involve the differentiation of specialized hyphae in response to starvation for nitrogen and/or carbon. In the life cycle of N. crassa, both asexual and sexual sporulation require differentiation of a vegetative mycelium, and both are induced by nutrient deprivation (26, 35, 45). Upon carbon starvation, the vegetative mycelium undergoes a process whereby specialized aerial hyphae form, grow away from the substrate, and produce aerial constrictions chains by repeated apical budding. When subject to nitrogen starvation, vegetative hyphae aggregate into a coiled body and form the spherical female reproductive structure (protoperitheciun) with specialized receptive hyphae (trichogynes).

In the yeast S. cerevisiae, filamentous differentiation is promoted by activation of a mitogen-activated protein kinase (MAPK) cascade pathway (27) in response to starvation for nitrogen, carbon, or other nutrients (14, 15, 25). The filamentous growth pathway is profoundly intertwined with the yeast mating response pathway (10, 34, 39). In analogy to yeast, the poi-2 gene may encode a component that is shared by the filamentous growth MAPK cascade and the pheromone response MAPK cascade pathways.

The poi-2 gene has an unusual structure, with 16 tandem repeats of 39 or 42 nucleotides, which constitute over 90% of the gene (Fig. 1). The tandem repeat structure of POI2, with each repeat flanked by Kex2 protease recognition sites, resembles that of the Rep1 precursor of Ustilago maydis (50) and the mating factor α (Mfa) pheromone precursor of S. cerevisiae (22). The processed Rep1 is fairly hydrophobic, and it plays a structural role in the formation of aerial hyphae and enhancement of surface hydrophobicity. Disruption of the Ustilago rep1 gene does not affect viability but greatly reduces surface hydrophobicity of haploid mutant strains. When two mutants are crossed, aerial growth and surface hydrophobicity of dikaryotic filaments are greatly reduced as well (50). The processed Mfa is hydrophilic, and it is required for initiation of the mating response MAPK pathway. Recently, evidence for posttranslational processing of POI2 has been obtained using vegetative (VM) plate cultures (Phillips et al., personal communication), which might explain the failure to detect POI2 in starved mycelia.

The Neurospora poi-2 gene appears to be essential, as no true knockout mutants were obtained. All poi-2 mutants had a coding region maintained in frame, despite numerous point and insertion/deletion mutations. The viability of sexual progeny was greatly reduced. Also, the poi-2 indel mutations resulted in severe vegetative and sexual growth defects, even though the mutants had a nearly full-length coding region without early chain terminators or frameshifts. Thus, a knockout of the poi-2 gene is predicted to be a lethal event.
In attempts to disrupt the poi-2 gene by RIP, several poi-2 strains with an unusual disruption of the gene (indel mutations) were isolated, in addition to the typical RIP mutants. Although they have little resemblance to the usual RIP-mediated changes, the changes in the indel progeny may be caused by a RIP-related process. In a gene lacking repeats, RIP occurs processively on a single strand of the DNA (defined here as the Watson strand), changing C to T; the Crick strand will remain unchanged until the next replication occurs (43). The mutations on the Watson strand (Watson-RIP) will tend to destabilize the double helix so that the Watson and Crick strands will have some tendency to separate, reassociate, and separate again (5). When they reassociate, the strands will do so correctly because there are no internal repeats competing for the correct pairing. In poi-2, however, the gene has 16 repeats, so in principle, there are 15 wrong ways to reassociate. Unless the association constant for perfect reassocation of the Watson-RIP strand with the Crick-non-RIP strand is much more than 15 times larger than that of the sum of all the wrong ways to pair, mispairing will occur. This will give rise to single-stranded loops in both the Watson-RIP and the Crick-non-RIP strands, each loop containing an integral number of repeat units. There are probably few thermodynamically preferred resting places for the single-stranded loops, and these would give rise to one or a few majority types of final events. This might explain why the eight indel alleles were identical, although they were isolated from two independent RIP crosses.

The distinctive RFLP pattern of poi-2 indels was not detected in the parental strains. However, the possibility that the indel mutations were present in a small number of the parental nuclei before the RIP cross cannot be excluded; preexisting indels would remain undetected if nuclei carrying the mutations represented a small fraction of the multinucleate parental strain.

In summary, the poi-2 gene appears to be essential and play a complex role in the life cycle of N. crassa. It is required for normal vegetative growth, female fertility, perithecial development, and production of ascospores. The novel poi-2 gene has been implicated in the mating response, probably as a component in the pathway rather than as a pheromone.

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