

A Mitogen-Activated Protein Kinase Pathway Essential for Mating and Contributing to Vegetative Growth in *Neurospora crassa*

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ABSTRACT

MAP kinases homologous to *Saccharomyces cerevisiae* Fus3p/Kss1p have been identified in plant pathogenic fungi and are required for pathogenicity and sexual reproduction. To better understand the role of MAP kinase signaling in *Neurospora crassa*, and to identify downstream target genes of the pathway, we isolated, cloned, and disrupted the *FUS3* homolog *mak-2*. Ste12p is a transcription factor target of Fus3p that activates genes of the mating pathway in yeast, and we also characterized the *N. crassa* *STE12* homolog *pp-1*. The *mak-2* and *pp-1* mutants have reduced growth rate, produce short aerial hyphae, and fail to develop protoperithecia. In addition, ascospores carrying null mutations of either gene are inviable. Subtractive cloning was used to isolate genes having reduced expression in the *mak-2* mutant. Expression of some of these genes is protoperithecia specific and three of them are part of a gene cluster potentially involved in the production of a polyketide secondary metabolite. Microarray analysis was used to extend the analysis of gene expression in *mak-2* and *pp-1* mutants. The role of the MAP kinase pathway in both sexual and asexual development as well as secondary metabolism is consistent with the dual regulation of the mating process and pathogenicity observed in fungal pathogens.

A family of serine/threonine protein kinases, known as the mitogen-activated protein (MAP) kinases, is involved in extracellular signal perception during growth and differentiation processes in eukaryotic organisms. In the unicellular yeast, *Saccharomyces cerevisiae*, five MAP kinase signal transduction pathways that regulate mating, filamentation, cell integrity, the response to high osmolarity, and ascospore formation have been characterized (GUSTIN *et al.* 1998). Fus3p MAP kinase controls the transduction of the pheromone signal in haploid cells, while Kss1p regulates nitrogen starvation-induced filamentous growth in diploid cells (GUSTIN *et al.* 1998; BREITKREUTZ and TYERS 2002). Homologs of *FUS3/KSS1* have been characterized in several pathogenic filamentous ascomycetes and play key roles in infection structure (appressorium) formation and host colonization (XU 2000). For example, in *Magnaporthe grisea*, the Pmk1p MAP kinase is required for production of female reproductive structures, appressorium development, and host colonization (XU and HAMER 1996).

The homologs of the *S. cerevisiae* transcription factor Ste12p, which is regulated by Fus3p and Kss1p, were also characterized in *M. grisea*, *Aspergillus nidulans*, and several other fungi (LIU *et al.* 1994; CHANG *et al.* 2000, 2001; VALLIM *et al.* 2000; YOUNG *et al.* 2000; BORNEMAN *et al.* 2001; PARK *et al.* 2002). In *A. nidulans*, *steA* is required for sexual development (VALLIM *et al.* 2000). In *M. grisea*, *MST12* was shown to function downstream of *PMK1* in the regulation of host penetration and invasive growth, but was not required for appressorium formation (PARK *et al.* 2002). Interestingly, *MST12* was not required for female sexual fertility (PARK *et al.* 2002), providing a dramatic example of divergence in the role of *MST12* in *M. grisea* compared to the *A. nidulans* counterpart. *Neurospora crassa* is a well-characterized model organism ideally suited to examining the role of MAP kinase function in mating behavior and development. Studies of the heterotrimeric G-proteins in *N. crassa* reveal a role in aerial hyphae formation and asexual sporulation (KAYS and BORKOVICH 2004) and additional roles in protoperithecium function (KIM and BORKOVICH 2004). Because *N. crassa* is more closely related to *M. grisea* than is *A. nidulans*, determining the role of the ortholog of *MST12/steA* in *N. crassa* is of interest for understanding the evolutionary steps leading to the functional difference between *MST12* and *steA*.

We characterized *N. crassa* homologs of *S. cerevisiae* *FUS3/KSS1* and *STE12* genes, called *mak-2* (mitogen activated kinase-2) and *pp-1* (protoperithecium-1), respectively. Both genes are essential for protoperithecia for-

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mation and contribute to maintaining normal filamentous growth and development of aerial hyphae. Several downstream targets genes of the MAP kinase pathway, which were called *mak-2* kinase-regulated genes (*mkr*), were isolated and studied. A gene encoding a polyketide synthase and three *mkr* genes were found to be clustered and coregulated during protoperithecia and fruiting body formation. This finding suggests the possibility that genes involved in plant pathogenesis may have been coopted from the mating process by adaptation of this MAP kinase pathway involved in regulating both morphogenesis and secondary metabolism.

MATERIALS AND METHODS

Strains, culture conditions, and fungal transformation: *N. crassa* strains 74-OR23-1VA (FGSC 2489), 74-ORS6a (FGSC 4200), and *cr-1* mutant (FGSC 4345) were obtained from the Fungal Genetics Stock Center (FGSC, Kansas City, KS). Vogel's minimal (VM) medium and synthetic crossing (SC) medium were prepared as described (DAVIS and DE SERRES 1970). For RNA extraction, conidia were collected in sterile water from flasks with VM solid medium after 7 days of incubation at 34° and filtered through Miracloth (Calbiochem, La Jolla, CA). Cultures were inoculated with 10⁶ conidia/ml and grown in flasks at 34° at 250 rpm. Mycelia were harvested by filtration onto filter paper in a Bücher funnel. Mycelia from cellophane overlays on VM and SC plates were collected after 7 days of incubation at 34° or 25°, respectively.

For microarray experiments, to reduce the variation arising from an individual growth experiment, mycelia were harvested from 12 independent SC plates (a pair of plates inoculated on each of 6 successive days) for total RNA extraction. RNA from cultures harvested each day were isolated separately and combined in equal amounts for labeling prior to microarray hybridization.

Growth rate was measured on race tubes with VM solid medium (DAVIS and DESERRES 1970). The experiment was repeated three times and the average growth rates were calculated. Crosses were performed on SC medium, and random ascospores were picked and germinated as described previously (PERKINS and RAJU 1986). To examine the effect of cAMP on production of aerial hyphae, 2 mM cAMP (Sigma, St. Louis) was added to standing VM liquid cultures as described previously (KAYS *et al.* 2000; IVEY *et al.* 2002). To test for conidiation in submerged liquid cultures, conidia were inoculated at a concentration of 1 × 10⁶ conidia/ml into 50 ml of SC medium, SC with 2% peptone, VM, and VM with 2% peptone and grown as described above.

N. crassa was transformed by electroporation of conidia on the basis of a published protocol (MARGOLIN *et al.* 1997). The transformed conidia were then mixed with 45 ml of the regeneration agar [VM with 3% sorbose, 0.05% glucose, 0.05% fructose (FGS) as carbon source, 1M sorbitol, 1.5% agar] at 55°, poured onto VM FGS (DAVIS and DE SERRES 1970) agar plates with an appropriate concentration of selective agent, and incubated at 34°.

Nucleic acid manipulations: All nucleic acid manipulations were performed using standard methods (SAMBROOK and RUSSELL 1989). Genomic DNA was isolated from *N. crassa* mycelia as described (VOLLMER and YANOFSKY 1986). RNA isolation and Northern blot analyses were carried out as described (SACHS and YANOFSKY 1991). Northern blot hybridizations were performed according to the manufacturer's protocol (Zeta-Probe Blotting Manual, Bio-Rad Laboratories, Hercules,

CA). Probe labeling was performed using the Rediprime II system labeling kit from Amersham Pharmacia Biotech (Piscataway, NJ). A phosphorimager (Fuji BAS-1800 II) was used to quantitate signals on blots for comparison to microarray results. To analyze the expression pattern of the polyketide synthase gene (NCU02918.1) (GALAGAN *et al.* 2003), reverse-transcription-polymerase chain reaction (RT-PCR) was used. First-strand cDNA was generated from the total RNA using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). The double-stranded cDNA was then amplified by PCR with the specific primers PDL21 (Table 1) and PDL22 to produce the 661-bp PCR fragment specific to NCU02918.1. *N. crassa actin* (NCU04173.1) specific primers, actin1 and actin3, were used to amplify *actin* cDNA as a control.

Four cosmids containing the *mak-2* gene were identified: G13A3, G14H8, X1E10, and X15B5. To subclone the *mak-2* gene, cosmids G14H8 and X15B5 were digested with selected restriction enzymes and subjected to Southern analysis. Three DNA fragments that gave a positive signal when probed with *PMK1* cDNA were isolated and cloned into the vector, pCB1004 (SWEIGARD *et al.* 1997): a 2.5-kb *Bgl*II-*Bgl*II (pBP26), a 7.5-kb *Bam*HI-*Bam*HI (pMB1), and a 2.2-kb *Bam*HI-*Bam*HI (pMB2). These clones were used as template DNA to sequence the *mak-2* locus. The coding region of the *mak-2* gene was PCR amplified from *N. crassa* cDNA with primers MTH-1 and MTH-2 and then sequenced to verify its integrity.

A cDNA clone (NCW10A9) containing a region of homology to the *A. nidulans* SteA protein was identified by sequence alignment (NELSON *et al.* 1997). A portion of this cDNA was amplified by PCR with STE-1 and STE-2 primers and used as a probe to screen the *N. crassa* pMOCosX cosmid library (ORBACH 1994). Four cosmids were identified in the screen: X17D4, X3B2, G21F5, and G21G11. The complete sequence of the gene was determined and we named the gene *pp-1* (*proto*perithecia-1) because the phenotype of the corresponding mutant strain resembled the previously described *pp* mutant phenotype (PERKINS *et al.* 2001). The coding region of the *pp-1* gene was PCR amplified from *N. crassa* cDNA with primers STH-1 and STH-2 and then sequenced to compare with the genomic sequence to verify predicted intron locations.

A *mak-2* gene replacement vector pBP-KOMAK2 was constructed in the pCB1532 vector (SWEIGARD *et al.* 1997). First, an *Eco*RI-*Hind*III *hph* cassette was cloned into the *Eco*RI-*Hind*III polylinker sites of pCB1532 to create pBP27. Next, the 2.2-kb *Bam*HI-*Bam*HI fragment (from pMB2) containing the *mak-2* downstream flanking region was ligated into the *Bam*HI site of pBP27. The 2-kb *Xho*I-*Xho*I upstream flanking region of *mak-2* was PCR amplified from plasmid pBP30 with T7 and MAK1 (containing an *Xho*I site) primers and ligated into the *Xho*I site to produce pBP-KOMAK2. Before transformation into *N. crassa* 74-ORS6a, pBP-KOMAK2 was linearized with *Not*I. Hygromycin-resistant transformants were isolated and screened by PCR using primers PDL37 and PDL38. Isolates of each *mak-2* knockout mutant were purified to homokaryons by three rounds of conidial plating and then confirmed by Southern blot analysis. The *mak-2* complementation vector was generated by cloning a 5.6-kb *Eco*RI-*Hind*III fragment from the cosmid G14H8 (-2212 to +3412 bp with respect to the predicted start codon of *mak-2*) into the corresponding restriction sites of pCB1532. The resulting plasmid pDL3 was used to transform conidia of the *mak-2* strain with selection for sulfonyleurea resistance (100 µg/ml chlorimuron ethyl). Then the sulfonyleurea-resistant transformants were screened for the presence of the *mak-2* coding region by PCR using PDL37 and PDL38 primers.

The *pp-1* gene replacement plasmid pBP-KOSTE12 was constructed in pBluescript SK vector (Stratagene, La Jolla, CA). First an *Hpa*I-*Hpa*I *hph* cassette from plasmid pCB1004 was

TABLE 1
Primers, strains, and plasmids used in the study

Primers	Sequences
PDL21	5'-GTCGATGTCGTCCTCAACTTTGCCAAC-3'
PDL22	5'-GGTCCATCGATAACCATTGACAGCCAAC-3'
actin1	5'-GTCGTA CTCTGCTTGGAGATCCACAT-3'
actin3	5'-CTCAGTCCAAGCGTGGTATCCTTACTC-3'
MTH-1	5'-GTCGAATT CAGCAGCGCACAAAGAGGC-3'
MTH-2	5'-ACACGGATCCTCATAATCTCCTGGTAG-3'
STE-1	5'-TCAACGATGACGAACTTGC-3'
STE-2	5'-ACGCTTGTGCTGTGCTAGG-3'
STH-1	5'-CTTGAATTCTATTCTTCGCAGCATGCC-3'
STH-2	5'-ACTGGATCCCCTTCGCATCATCTCGC-3'
MAK1	5'-TTCCTCGAGTCCCGAATAGTTGCTTGC-3'
T7	5'-GTAATACGACTCACTATAGGGC-3'
PDL37	5'-CAAGCAACTTTTCGGGAC-3'
PDL38	5'-ATCGGTAGACACTTCACC-3'
STE3	5'-TCCAGACTTTGAGCATGC-3'
STE3KO	5'-TAATGCAAGTCCGATTCCG-3'
STEK4	5'-TGGCACAGATATGTGAGCAGTTCTG-3'
CTRP2	5'-CGCACCAAGTTATCGTGCACCAAGC-3'
STE11	5'-ACCGCAATCGTTTCGTGC-3'
STE14	5'-AGCGTGGAACTATAGTAAG-3'
SE1	5'-TAGTCCGAATCAAGCAAGAGCACA-3'
SE2	5'-CTCTTGCTTGAATTCGGACTA-3'
Strains	Genotype/comment
74-OR23-1VA	Wild-type strain, <i>mat A</i> (FGSC 2489)
74-ORS6a	Wild-type strain, <i>mat a</i> (FGSC 4200)
PBM5	Δ <i>mak-2 a</i> , constructed in 74-ORS6a
PBMP5-1	Δ <i>mak-2 A</i> , F1 progeny of PBM5 cross with 74-OR23-1VA (FGSC 9353)
PBM7	Δ <i>mak-2 a</i> , constructed in 74-ORS6a
PBM49	Δ <i>mak-2 a</i> , constructed in 74-ORS6a
DL14	Δ <i>pp-1 A</i> , constructed in 74-OR23-1VA (FGSC 9352)
FGSC 4345	<i>cr-1, mat a</i>
Plasmids	Comment
pBP-KOMAK2	<i>mak-2</i> gene replacement plasmid
pBP-KOSTE12	<i>pp-1</i> gene replacement plasmid
pDL3	<i>mak-2</i> gene complementation plasmid
pBP15	pBluescript SK ⁻ vector with an <i>hph</i> cassette in the <i>EcoRV</i> site
pBP26	pCB1004 with a 2.5-kb <i>Bgl</i> II fragment of cosmid G14H8 in the <i>Bam</i> HI site
pBP27	pCB1532 with an <i>Eco</i> RI- <i>Hind</i> III <i>hph</i> cassette
pBP30	pCB1004 with a 2-kb <i>Xho</i> I upstream flanking region of <i>mak-2</i>
pMB1	pCB1004 with a 7.5-kb <i>Bam</i> HI fragment of cosmid G14H8 in the <i>Bam</i> HI site
pMB2	pCB1004 with a 2.2-kb <i>Bam</i> HI downstream flanking region of <i>mak-2</i>

cloned into the *EcoRV* site of pBluescript vector. The resulting plasmid was named pBP15. Next, a 1.7-kb *pp-1* downstream flanking region was PCR amplified using STE3 and STE3KO primers and cloned into the TOPO vector (Invitrogen, Carlsbad, CA). The *Eco*RI fragment containing the downstream flanking region was cloned into the *Eco*RI site of pBP15. The 1.6-kb *Kpn*I-*Hind*III upstream flanking region of *pp-1* was obtained from cosmid X17D4 and cloned into the *Kpn*I-*Hind*III site of pBP15 to create plasmid pBP-KOSTE12 plasmid. Before transformation into *N. crassa*, pBP-KOSTE12 was digested with *Not*I and *Kpn*I enzymes to release the cloned DNA from the vector sequences. Hygromycin-resistant transformants were isolated and screened by PCR using primers STEK4 and

CTRP2, and the deletion of *pp-1* gene was confirmed by PCR with primers STE11 and STE14. Homokaryons for each *pp-1* knockout mutant were obtained as described above for *mak-2* mutants.

Subtractive cloning of *mak-2*-regulated genes: To clone genes differentially expressed in the *mak-2* mutant, a subtractive enrichment protocol described by WANG and BROWN (1991) was followed. Both the wild-type (74-ORS6a) and *mak-2*-knockout (PBM5) strains were grown at 25° with constant illumination on SC plates overlaid with cellophane. After 6 days, protoperithecia were well developed in the wild-type strain. Mycelia of both strains were collected and used for isolation of total RNA. Next, poly(A)⁺ RNA was purified (PolyATrack mRNA

Isolation Systems, Promega, Madison, WI) from ~1.5 mg of total RNA, and 1 μ g of poly(A)⁺ RNA was used to synthesize cDNA (cDNA Synthesis Kit, Amersham Pharmacia Biotech, Piscataway, NJ). cDNAs were digested with *Rsa*I to produce blunt-ended fragments. Fragments in the size range of 100–500 bp were purified from the agarose gel and ligated with double-stranded linkers SE1 and SE2. cDNA fragments were subsequently amplified by PCR with the single primer SE2. To isolate genes downregulated in the *mak-2* mutant, biotin was incorporated into the cDNA from the *mak-2*-mutant strain and this biotinylated cDNA was used in excess to hybridize to cDNA from the wild-type strain. The common sequences present in the wild-type strain that hybridized with biotinylated cDNA of the mutant were subtracted by binding with streptavidin followed by phenol extraction. After two rounds of subtractive enrichment, the remaining cDNA fraction was cloned into the pBluscript SK⁻ vector after *Eco*RI digest. A library of *Escherichia coli* transformants was used to prepare colony blots and hybridized with cDNA probes prepared from wild-type and *mak-2*-mutant RNA. Clones hybridizing with the wild-type cDNA but not with the *mak-2*-mutant cDNA were sequenced and gene expression was analyzed by Northern blotting.

Microarray hybridization and data analysis: The microarray chips and hybridization conditions were identical to those described previously (LEWIS *et al.* 2002; XIE *et al.* 2004). In brief, the microarray consisted of 3840 features composed of 1920 pairs of spots including 1764 ESTs. Additional control samples included plasmid DNA and PCR products for known *N. crassa* genes. The microarray represents 1335 unique genes or ~13% of all predicted *N. crassa* genes (LEWIS *et al.* 2002; XIE *et al.* 2004). The two-channel (Cy3 and Cy5) labeling system, which allows simultaneous comparison of gene expression patterns, was used (DERISI *et al.* 1997). cDNA samples of wild type (74-OR23-IVA), $\Delta pp-1$ (DL14), or $\Delta mak-2$ (PBMP5-1) were generated by reverse transcription from 400 ng mRNA starting material. Reverse transcription and subsequent hybridization of the microarray followed the two-step protocol of the 3DNA Submicro EX expression array detection kit (Genisphere, Hatfield, PA) as described previously (XIE *et al.* 2004). Fluorescence images were acquired using an Affymatrix 426 array scanner (Affymatrix, Santa Clara, CA) and then imported into ScanAlyze (<http://rana.lbl.gov/EisenSoftware.htm>) to generate raw signal intensities for each spot. The *N. crassa* microarray data files are available at <http://plpa2linux.tamu.edu/microarray.html>.

XIE *et al.* (2004) compared three statistical approaches for the analysis of the effects of glucose on *N. crassa* gene expression using an identical microarray. That study indicated that Student's *t*-test for significance (Genespring, Silicon Genetics, Redwood City, CA), which determines whether the mean normalization value is significantly different from 1.0, is the most sensitive approach, as compared to MARAN (ENGELEN *et al.* 2003) and SAM (TUSHER *et al.* 2001). Given the fact that, as expected, the relative differences between mutants and wild type in this study tend to involve fewer genes on the array and, also, tend to be exhibited as a lesser fold change difference than those in the glucose comparison, we used Genespring version 6.1 (Silicon Genetics) for this analysis. Furthermore, since we were interested not only in the difference in each mutant *vs.* wild type, but also in a direct comparison of the two mutant responses relative to the wild-type controls, the availability of the ANOVA and Welch *t*-test features to conduct these comparisons also justified the choice of Genespring 6.1.

Six or eight data points per gene (duplicate spots on each of three or four independent arrays per mutant studied) were taken. Prior to normalization, the background value for each spot was subtracted from the raw intensity value. The data were then normalized by the per spot and per chip intensity-

dependent (Lowess) procedure using the default settings. Because measurements with higher control signal strength are relatively more precise than measurements with lower control signal strength, the cross-gene-error model in Genespring calculates a cutoff control intensity value for "reliable" data. Thus, the first filter we applied to the normalized data was based on this cutoff control signal strength. To consider only the genes reliable in both groups, the filter was applied to screen for genes with control signals above the cutoff value in two of two conditions ($\Delta mak-2$ *vs.* wild type and $\Delta pp-1$ *vs.* wild type). Next, to identify genes regulated by either *mak-2* or *pp-1* or by both, this list of reliable genes was filtered for genes with Student's *t*-test *P*-value <0.05 in one of two conditions. These *t*-tests were calculated with the Benjamin and Hochberg false discovery rate multiple-testing correction. The final filter applied to limit the number of regulated genes for further analysis was based on an arbitrary fold change (Signal/Control) of at least twofold (decrease or increase) in mRNA levels in either of the mutants relative to the wild type.

To compare the effect of the *mak-2* and the *pp-1* mutations on gene expression, we applied one-way analysis of variance (ANOVA) to genes that were regulated at least at twofold. The Benjamini and Hochberg false discovery rate multiple-testing correction was applied during this procedure. Since the distribution of values for genes whose expression decreased twofold or more were bounded between 0 and 0.5, we assumed that the variances were not equal for the comparison of these genes. Thus, the test of significance was actually the Welch *t*-test, a common nonparametric substitution made when the assumptions of ANOVA are not met. In contrast, for comparison of the genes whose expression increases at least twofold relative to wild type, the variances were calculated using the cross-gene-error model and on-chip replicates and a standard ANOVA was applied.

RESULTS

Cloning and sequence analysis of the *mak-2* and *pp-1* genes: The *mak-2* gene encoding the *N. crassa* homolog of yeast Fus3/Kss1 MAP kinases was identified on the basis of sequence similarity to the *M. grisea* *PMK1* gene. The nucleotide sequence of the cloned DNA was determined and revealed an open reading frame (ORF) of 1278 bp that codes for a protein of 352 amino acids corresponding to NCU02393.1 (GALAGAN *et al.* 2003). The coding region of the *mak-2* gene is interrupted by three introns. The presence of the introns was confirmed by sequencing the *mak-2* cDNA fragment amplified from a *N. crassa* cDNA library by PCR with primers designed to amplify the entire coding sequence. The predicted amino acid sequence of the *mak-2* protein demonstrated high identity to other fungal MAP kinases and contains 11 conserved protein kinase domains, as well as the TEY dual phosphorylation motif specific for this group of MAK kinases (KULTZ 1998; PANDEY *et al.* 2004).

To isolate the *N. crassa pp-1* gene, a genomic cosmid library was screened with a PCR-amplified cDNA fragment that showed similarity to the *A. nidulans steA* gene (see MATERIALS AND METHODS). The sequence of the identified ORF encodes a protein of 722 amino acids and is interrupted by three introns that correspond to

NCU00340.1 (GALAGAN *et al.* 2003). The position of the predicted introns was confirmed by sequencing a PCR product obtained by using a cDNA library as template. The *N. crassa pp-1* protein shows a high identity to orthologs from other fungi. Similar to *A. nidulans* SteAp and *M. grisea* Mst12p, the N-terminal part of the protein contains a conserved homeodomain and the C-terminal region contains two Cys2/His2 zinc finger motifs. The overall sequence identity to Ste12p orthologs from yeasts is low and the yeast proteins lack the zinc finger domain. Additionally, BLAST search against the Protein Data Bank (PDB) showed a conserved proline at amino acid position 101 of PP-1, which might be a potential MAP kinase-binding site (OBENAUER *et al.* 2003) (supplementary Figure S1 at <http://www.genetics.org/supplemental/>).

Deletion of *mak-2* and *pp-1*: To determine the roles

of the *mak-2* and *pp-1* genes we generated deletion mutants by homologous recombination. The disruption vectors were constructed by replacing the ORFs with a hygromycin-resistance marker (Figure 1, A and B). The *mak-2* construct removes nucleotides starting 8 bp upstream of the *mak-2* start codon to 278 bp downstream of the stop codon. The construct was transformed into the *mat a* strain 74-ORS6a and the correct integration confirmed by Southern hybridization (Figure 1C). We identified three *mak-2*-knockout strains: PBM5, PBM7, and PBM49. A 1.5-kb *KpnI* fragment overlapping the *mak-2*-coding region was used as a hybridization probe to verify the deletion. This probe identified a 7.5-kb *BamHI* fragment in the wild type that was absent in the $\Delta mak-2$ strains (Figure 1C, left). Likewise, the 1.5-kb *KpnI* band was absent because of the deletion of the *KpnI* site, leaving a 9.0-kb *KpnI* fragment in the $\Delta mak-2$ mutants. When the 1.4-kb *hph* gene was used as a probe, the predicted 7.5-kb *BamHI* and 9-kb *KpnI* fragments were observed in the $\Delta mak-2$ mutants (Figure 1C, right). The PBM5 strain was then backcrossed with 74-OR23-1VA, and a *mak-2::hph mat A* progeny was isolated and named PBMP5-1. The phenotypes of PBM5 and PBMP5-1 were indistinguishable.

For *pp-1*, a deletion starting 4 bp upstream of the start codon and ending 82 bp upstream of the stop codon was generated (Figure 1B). A *pp-1* knockout strain, DL14, was constructed in the 74-OR23-1VA background (Figure 1D). A 1.6-kb *KpnI-HindIII* upstream flanking region of *pp-1* (Figure 1B) was used as a hybridization probe. The wild type produced the expected *XhoI* fragments (1.7 and 0.9 kb) and a 2.3-kb *KpnI* fragment, whereas in the knockout mutant, the 0.9 *XhoI* fragment was converted to a 4.2-kb *XhoI* fragment due to deletion of the *XhoI* site in the mutant and the 2.3-kb *KpnI* fragment was converted to an 8.4-kb fragment due to removal of a *KpnI* site by the deletion (Figure 1D, left). When the 1.4-kb *hph* gene was used as a probe, the knockout mutant showed a 4.2-kb *XhoI* band and a 8.4-kb

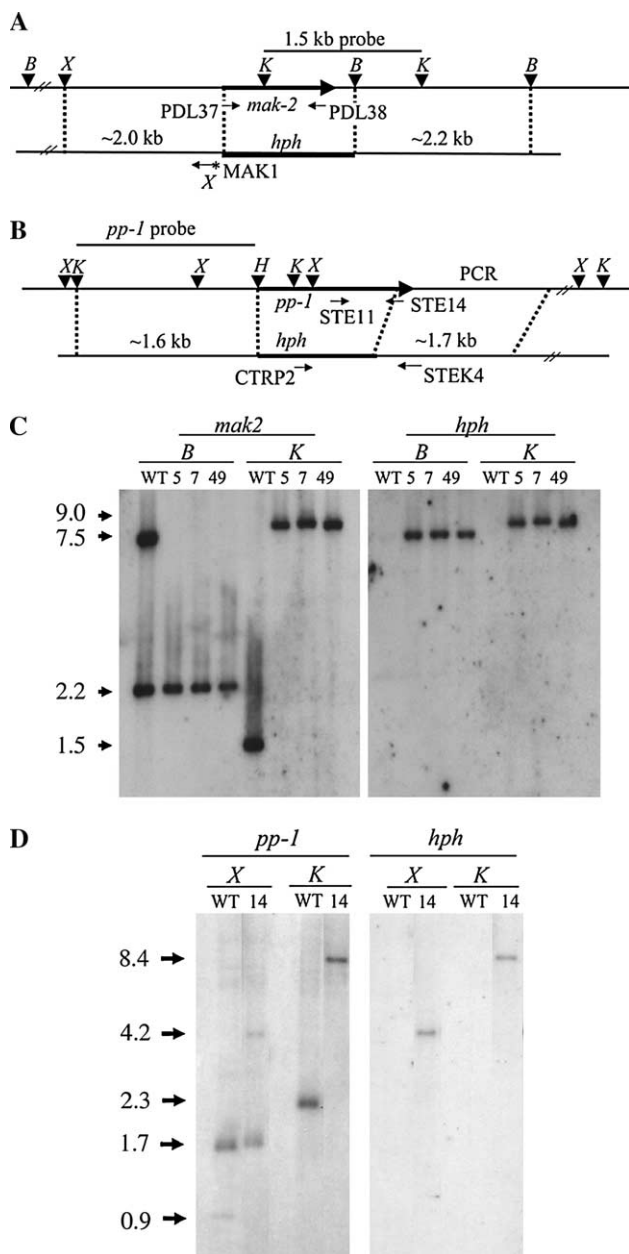


FIGURE 1.—Construction of *mak-2* and *pp-1* gene replacement mutants. (A) Physical map of the *mak-2* genomic region and gene replacement vector pBP-KOMAK2. The *XhoI* site introduced from primer MAK1 is indicated with an asterisk. PDL37 and PDL38 are primers used to screen $\Delta mak-2$ knockout mutants. The indicated 1.5-kb *KpnI* fragment was used as a hybridization probe for Southern blot analysis. The restriction sites are X, *XhoI*; K, *KpnI*; H, *HindIII*; B, *BamHI*. (B) Physical map of *pp-1* genomic region and gene replacement vector pBP-KOSTE12. STE11, STE14, STEK4, and CTRP2 are primers used to screen $\Delta pp-1$ knockout mutants. (C) Southern analysis of $\Delta mak-2$ knockout mutant strains PBM5, PBM7, and PBM49. Genomic DNAs were digested with *BamHI* (B) or *KpnI* (K) yielding fragments of the indicated sizes (kb). (D) Southern analysis of the $\Delta pp-1$ knockout mutant strain DL14. Genomic DNAs were digested with *XhoI* (X) or *KpnI* (K) to yield fragments of the indicated sizes.

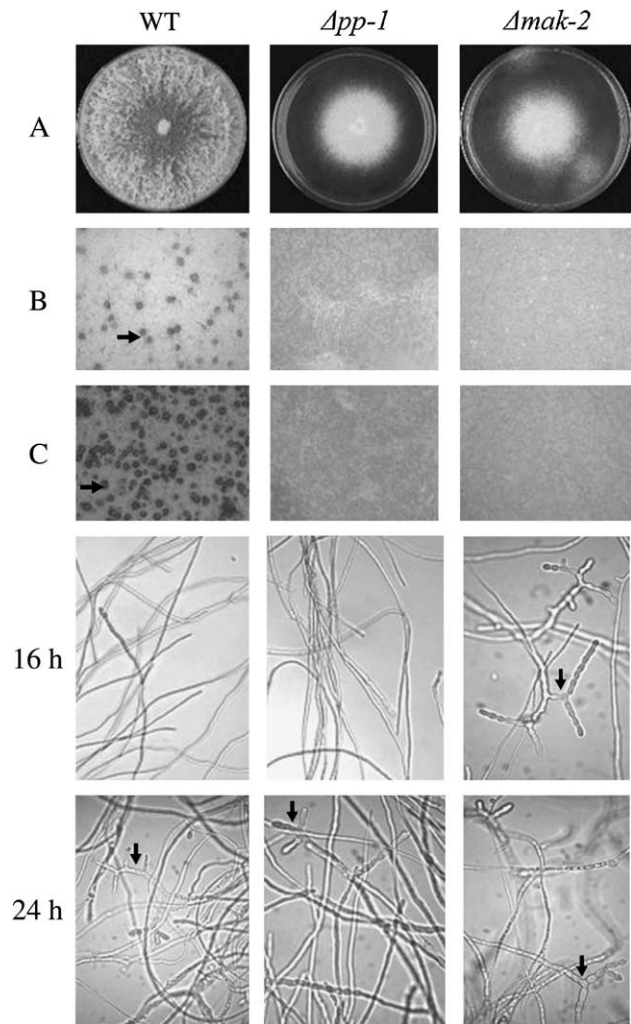


FIGURE 2.—Phenotypes of wild-type (WT), $\Delta pp-1$, and $\Delta mak-2$ isolates. (A) Colony growth on VM plates after 4 days at 34°. (B) Protoperithecia (arrow) formation on SC plates after 7 days at 25°. No protoperithecia were observed in the mutants. (C) Perithecia (arrow) development on SC plates after 7 days growth that were fertilized with WT (74-ORS6a) conidia overnight. No perithecia were observed in the mutants. Conidiation phenotype of WT (74-OR23-1VA), $\Delta pp-1$, and $\Delta mak-2$ strains in SC liquid medium. The cultures were grown at 34° with constant agitation at 250 rpm. At 16 hr of incubation, conidiophores were observed in the $mak-2$ mutant (arrow) but not in the $pp-1$ mutant or wild-type cultures. By 24 hr, conidiophore formation was observed in all strains (arrows), but was predominant for the $mak-2$ mutant.

KpnI band as expected (Figure 1D, right). We selected the PBMP5-1 and the DL14 isolate for further analysis.

Growth and development of $\Delta mak-2$ and $\Delta pp-1$ mutants: Mycelial elongation rates for both the $\Delta mak-2$ and $\Delta pp-1$ strains were 25% of the wild-type controls (Figure 2A), and these observations were verified in race tube growth experiments (3.8 mm/hr *vs.* 1.0 mm/hr). Both $\Delta mak-2$ and $\Delta pp-1$ mutants never formed protoperithecia even after extended incubation time (Figure 2B), nor could they be fertilized to form perithecia (Figure 2C). The inability to form protoperithecia accounts for

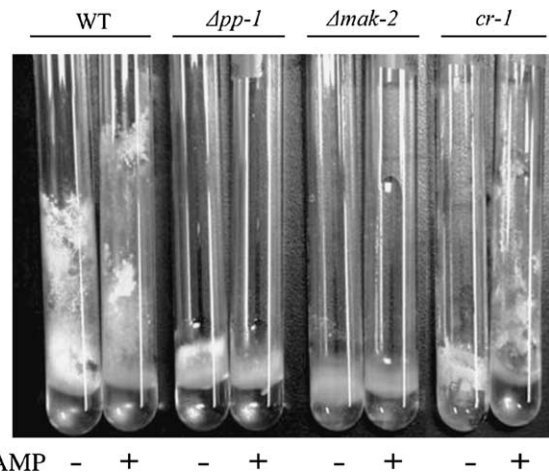


FIGURE 3.—Aerial growth of strains with and without 2 mM cAMP for wild-type, $\Delta pp-1$, $\Delta mak-2$, and $cr-1$ mutants. Aerial development of the $cr-1$, but not $pp-1$ or $mak-2$ mutants, was rescued by cAMP.

the complete female sterility of the mutants. However, the conidia of both $\Delta mak-2$ and $\Delta pp-1$ mutants were able to fertilize protoperithecia formed by the wild type. Protoperithecia from those crosses developed into mature perithecia with asci containing eight black ascospores. However, only about half of the isolated ascospores from the crosses were viable, and the vast majority of the ascospores that germinated developed into colonies displaying wild-type growth and sensitivity to hygromycin. The poor recovery of hygromycin-resistant progeny (<0.1%) indicates that $\Delta mak-2$ and $\Delta pp-1$ cause ascospore lethality. As mentioned above, a rare hygromycin-resistant progeny was obtained for the $mak-2$ mutant and this isolate was designated PBMP5-1. PBMP5-1 displayed all the phenotypes of the original mutant strain. To further demonstrate that all of the phenotypes of PBMP5-1 are due to mutation of $mak-2$, we complemented the mutant phenotype by transformation of PBMP5-1 (data not shown).

When grown on solid medium, both mutants produced abundant conidiophores similar in morphology to wild type, but they were produced very close to the surface due to the lack of normal aerial hyphae (Figures 2A and 3). The phenotype of reduced aerial hyphae is similar to that of the adenylate cyclase mutant $cr-1$ (IVEY *et al.* 2002; KAYS *et al.* 2000; YANG *et al.* 2002); therefore, we examined the effect of cAMP on aerial growth in standing liquid cultures. cAMP restored aerial hyphae production to the $cr-1$ mutant but not $\Delta mak-2$ or $\Delta pp-1$ (Figure 3). The $cr-1$ mutant also produces conidia prematurely in liquid cultures. We noted that when grown in SC liquid medium, the $\Delta mak-2$ -mutant cultures tended to produce carotenoid pigmentation associated with conidiation after 16 hr of incubation. Under the same conditions, the wild-type strain and the $\Delta pp-1$ mutant began to conidiate ~ 24 hr after inoculation (Figure 2).

TABLE 2
Summary of *mkr* genes obtained from subtractive enrichment

Gene	NCU no. ^a	Contig ^b	Expression pattern ^c	BLAST match (organism), <i>e</i> -value
<i>mkr1</i>	NCU08907.1	3.557	Unspecific	Related to blastomyces yeast phase-specific protein (<i>Ajellomyces dermatitidis</i>), 2e-10
<i>mkr2</i>	NCU02923.1	3.152	Protoperithecia specific	Putative short chain dehydrogenase (<i>S. probe</i>), 3e-12
<i>mkr3</i>	NCU02930.1	3.152	Protoperithecia specific	Putative pyridoxal reductase (<i>S. probe</i>), 7e-67
<i>mkr4</i>	NCU06597.1	3.382	Unspecific	Unknown
<i>mkr5</i>	NCU07449.1	3.434	Aerial hyphae specific	Unknown
<i>mkr6</i>	NCU02919.1	3.152	Protoperithecia specific	C terminus similar to <i>binA</i> gene product (<i>A. nidulans</i>), 5e-16

^a ORF ID numbers assigned by the Broad Institute (GALAGAN *et al.* 2003).

^b Contig ID of assembly three of the *N. crassa* genome (GALAGAN *et al.* 2003).

^c Based on Northern analysis (Figure 4A; data not shown).

These observations suggest that there is a moderate derepression of conidiation in the *mak-2* mutant.

Isolation of genes with *mak-2*-dependent expression:

In yeast, the Fus3p/Kss1p MAP kinase cascade regulates a number of genes involved in mating and invasive growth. In an attempt to isolate genes regulated by the putative *mak-2* kinase we performed subtractive enrichment (see MATERIALS AND METHODS). Because two major phenotypes of the Δ *mak-2* mutant, reduced aerial hyphae and female sterility, can be easily detected on SC solid medium, both the wild-type strain and the Δ *mak-2* mutant were grown on SC plates for 5 days at room temperature until the protoperithecia were formed in the wild type. After two rounds of subtractive enrichment, 46 cDNA fragments detected only in cDNA isolated from the wild-type strain were identified. Sequence analysis revealed that these correspond to six *mkr* genes (Table 2). The expression of *mkr* genes was analyzed in wild-type, Δ *mak-2*, and Δ *pp-1* mutants under different growth conditions by Northern hybridization. Three of these genes *mkr-2* (NCU02923.1), *mkr-3* (NCU02930.1), and *mkr-6* (NCU02918.1) are expressed preferentially in the wild-type strain grown on SC plates, conditions that induce protoperithecia development (Figure 4A). These three genes were found clustered on chromosome I (Figure 5). Analysis of the genomic sequence surrounding the *mkr-2*, *-3*, and *-6* genes revealed the presence of a paralog of *mkr-6* as well as an ORF encoding a putative polyketide synthase (*pks*) (NCU02918.1). The expression of the *pks* gene was most highly expressed in cultures grown on SC solid medium producing protoperithecia, or perithecia fertilized with conidia of opposite mating type, and this expression was dependent on *mak-2* and *pp-1* (Figure 4B).

Expression of the *mkr-5* (NCU07449.1) gene was detected only in the wild-type strain grown on solid medium (Figure 4A), a condition that produces aerial hyphae and conidia. Since *mkr-5* was not detected in isolated conidia, we conclude that *mkr-5* expression is limited to aerial hyphae and not expressed in hyphae submerged in liquid culture. Furthermore, *mkr-5* was

expressed at equally high levels in wild type and the aconidial *fl* mutant strain, supporting the view that it is expressed in aerial hyphae and not developmentally committed hyphae destined to form conidiophores. Interestingly, *mkr-5* is the first example of a gene in *N. crassa* with an “aerial hyphae-specific” transcript. Expression of the *mkr-1* and *mkr-4* genes was only moderately reduced in the *mak-2* mutant and varied from experiment to experiment (data not shown) and these genes were not characterized further.

Microarray analysis of gene expression in *mak-2* and/or *pp-1*: Seven microarray hybridizations were performed, four of which were replicates of the cDNA from the Δ *pp-1* mutant *vs.* the wild-type strain and three of which were replicates of the cDNA from the Δ *mak-2* mutant *vs.* the wild-type strain. The raw data were imported into GeneSpring version 6.1 to identify *mak-2* and/or *pp-1*-regulated genes (see MATERIALS AND METHODS). The cross-gene-error model identified 545.9 as the control strength cutoff intensity, which means that all genes with an average control signal less than this value were considered unreliable. As a result, 831 of 1920 (43.28%) individual pairs of features passed this filtering and were considered reliable. Among these reliable data, 280 of the 831 (34%) individual pairs of features displayed regulation by either *mak-2* or *pp-1* or by both (*t*-test *P*-value <0.05 in at least one of two conditions). To focus on the most highly regulated of these 280 individual pairs of features, 67 that showed at least a twofold change (36 increased and 31 decreased) in at least one of the two mutants relative to the wild-type strain were analyzed in greater detail.

These 67 ESTs represented 48 genes predicted from the *N. crassa* genome sequence and two ESTs (NM1H4 and NM5D2) that were ambiguous because the reported 5'- and 3'-end sequences for the cDNA clones represent different genes and these are likely to be chimeric (Table 3). On the basis of Northern blot analyses (data not shown), eight of the genes were removed from further consideration because their regulation was less than twofold. Genes were grouped into five classes on the

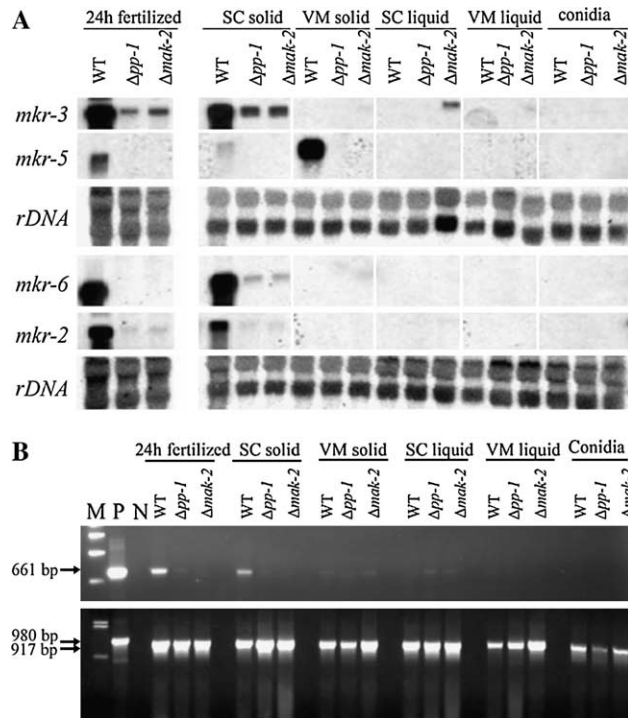


FIGURE 4.—Expression patterns of the *pks/mkr* gene cluster in the WT, $\Delta pp-1$ mutant, and $\Delta mak-2$ mutant. Strains were grown on SC solid medium for 7 days at 25° (SC solid), and then fertilized with 74-ORS6a conidia for 24 hr at 25° (24 hr fertilized), on VM solid medium for 7 days at 34° (VM solid), in SC liquid medium for 24 hr with 250 rpm shaking at 34° (SC liquid), in VM liquid medium for 24 hr with 250 rpm shaking at 34°. RNA was extracted from harvested cultures and from conidia. (A) Northern hybridization analysis. RNA blots were hybridized in succession with probes for *mkr-3*, *mkr-5*, *mkr-6*, and *mkr-2*. The blots were then hybridized with a rDNA probe to check the relative amount of RNA in each sample. (B) RT-PCR analysis of *pks* gene expression. PCR was performed with genomic DNA as a positive control (P) and no template as a negative control (N). The ΔDNA -*Bst*II digest ladder (New England Biolabs) was used to estimate the size of the PCR products (M). A 661-bp *pks* fragment (indicated by the arrow) was amplified with the gene-specific primers PDL21 and PDL22 using the first-strand cDNA as a template (top). The same cDNA samples were also used for RT-PCR with a primer pair specific for the actin gene, which was used as a control (bottom). The arrows indicate the PCR products amplified from gDNA (980 bp) and cDNA (917 bp).

basis of their microarray expression patterns (Tables 3 and 4). Class I included genes with expression significantly different from the wild type (*t*-test *P*-value < 0.05) for both mutants and with at least a twofold change relative to wild type in at least one mutant. These 25 unique genes required both *mak-2* and *pp-1* for maximal expression. Only one gene was assigned to class II and required *mak-2* but not *pp-1* for its expression (Table 3). No genes that were unambiguously regulated by *pp-1* but not *mak-2* were identified. Genes that displayed elevated expression in the mutants were also detected (Table 4). In class III, 12 genes displayed expression levels that were significantly different from the wild type

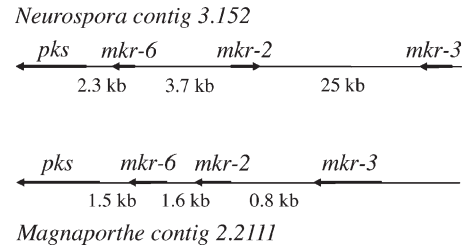


FIGURE 5.—Relative positions of genes in the *pks/mkr* gene cluster in *N. crassa* and an orthologous cluster in *M. grisea*. Arrows indicate the orientations of *mkr* genes. The distances between the ORFs are indicated.

(*t*-test *P*-value < 0.05) in both mutants and also displayed at least a twofold increase in expression in at least one of the mutants. Class IV included two genes in which expression was elevated in the *pp-1* mutant at least twofold but was unaffected or slightly downregulated in the *mak-2* mutant. The two genes in class V showed the reciprocal expression pattern.

ANOVA was applied to determine if the difference in regulation by *mak-2* and *pp-1* was significant (Tables 3 and 4). In most cases, genes in class I showed no significant differential regulation (*P*-value > 0.05) while the observed differential regulation displayed by the class IV and V genes were supported (*P*-value < 0.05). About half of the genes in class III also showed significant differential regulation (*P*-value < 0.05). Thus, the ANOVA analysis was consistent with the classification of gene expression assigned on the basis of expression ratios in conjunction with the *t*-test *P*-values. In addition, although *mak-2* and *pp-1* are both involved in repressing the expression of the class III genes, the magnitude of the regulatory effect of *mak-2* or *pp-1* differed significantly for some of the genes (Table 3).

Several genes were represented by more than one cDNA and in most cases displayed consistent results (Tables 3 and 4). Additionally, *mkr-3* was present on the microarray and was assigned as a class I gene as expected from Northern blot analysis. Northern blot analysis of representative genes was performed and expression ratios based on phosphorimager analysis were consistent with the microarray results (Tables 3 and 4).

DISCUSSION

Here we have isolated and characterized two genes regulating the mating pathway of *N. crassa*. The *mak-2* gene contains an open reading frame encoding a protein with 92.4% identity to Pmk1p, the ortholog of *S. cerevisiae* Fus3p. PP-1 shares 64.8% identity with Mst12p of *M. grisea*, particularly in the homeodomain in the N-terminal region (amino acids 60–204) and two conserved C₂/H₂-Zn²⁺ finger domains in the C-terminal region (amino acids 595–615, 621–643) (PARK *et al.* 2002). It is unknown if each of the two putative DNA-binding

TABLE 3
Genes with reduced expression in *mak-2* and *pp-1* mutants in microarray and Northern blot experiments

EST	Predicted ORF	Average fold change			BLAST match, <i>e</i> -value
		WT/ Δ <i>pp-1</i>	WT/ Δ <i>mak-2</i>	<i>P</i> -value ^a	
Class I					
NM6A4	NCU04931	2.9 (2.3)	2.8 (3.7)	0.838	Conserved hypothetical protein (<i>N. crassa</i>), 0.0
NP6A12	NCU04931	2.7	4.0	0.046	Conserved hypothetical protein (<i>N. crassa</i>), 0.0
NP2E11	NCU05236	6.3	7.0	0.738	Predicted protein (<i>N. crassa</i>), 1e-63
NM3D7	NCU05236	5.8 (33)	8.3 (50)	0.354	Predicted protein (<i>N. crassa</i>), 1e-63
NM1H4 (chimeric)	NCU08812; NCU03500	4.4 (ND)	5.9 (ND)	0.592	Conserved hypothetical protein (<i>A. nidulans</i>), 7e-56 Putative aminotransferase protein (<i>Ralstonia</i>), 5e-26
SP4A5	NCU07598	2.3 (ND)	1.9 (ND)	0.545	Related to salicylate 1-monooxygenase (<i>Emericella nidulans</i>), 5e-55; related to cysteine desulfurase NFS1, 1e-38
NM5F1	NCU04416	2.0 (4.1)	3.1 (3.3)	0.368	Conserved hypothetical protein (<i>N. crassa</i>), 0.0
NM5D2 (chimeric)	NCU07166; NCU04533	6.1 (ND)	4.7 (ND)	0.299	DNA-dependent RNA polymerase I (<i>S. cerevisiae</i>), 1e-16; Ca-dependent cell adhesion molecule-1 (<i>Dictyostelium</i>), 9e-11
NM7F7	NCU02988	6.7 (7.6)	3.9 (9.9)	0.298	Related to ubiquinone/menaquinone biosynthesis methyltransferase (COG2226.1, UbiE), 3e-05
NM8A5	NCU02259	6.1 (7.4)	3.4 (6.7)	0.294	Related to LST7 amino acid permease Golgi transport protein (<i>S. cerevisiae</i>), 2e-09
NM5C5	NCU04603	4.6 (6.9)	3.1 (12)	0.294	Riboflavin aldehyde-forming enzyme (<i>Agaricus</i>), 2e-08
W10D8	NCU07222	5.8 (16)	3.8 (14)	0.291	Conserved hypothetical protein (<i>M. grisea</i>), 2e-38
NM3H7	NCU08691	6.6 (6.3)	3.9 (10)	0.291	Conserved hypothetical protein (<i>M. grisea</i>), 3e-50
NM4G7	NCU01724	7.4 (3.8)	5.0 (4.9)	0.165	Related to Na ⁺ /H ⁺ -exchange protein (<i>S. cerevisiae</i>), 2e-66
NM8A11	NCU02532	4.2 (ND)	2.7 (ND)	0.165	Probable ubiquitin-activating enzyme (<i>N. crassa</i>), 0.0
SM3D2	NCU02930	11.9 ^c	4.9 ^c	0.165	<i>mkr-3</i> , pyridoxal reductase (<i>S. pombe</i>), 4e-67
NM1A10	NCU04088	7.4 (ND)	4.1 (ND)	0.165	Cell wall surface anchor family protein (<i>Streptococcus</i>), 7e-10
NM7F6	NCU05521	3.1 (ND)	2.2 (ND)	0.165	Pathogenicity protein (<i>PATH531</i>) mRNA (<i>M. grisea</i>), 9e-64
SP4D12	NCU05768	5.4	2.5	0.165	<i>poi-2</i> (<i>N. crassa</i>), 0.0
SM1B11	NCU05768	7.9	3.3	0.165	<i>poi-2</i> (<i>N. crassa</i>), 0.0
NM1A11	NCU05768	7.5 (7.3)	6.0 (9.0)	0.165	<i>poi-2</i> (<i>N. crassa</i>), 0.0
NM1F7	NCU05768	6.3	3.5	0.165	<i>poi-2</i> (<i>N. crassa</i>), 0.0
SP4F9	NCU05768	3.2	1.4	0.010	<i>poi-2</i> (<i>N. crassa</i>), 0.0
NM4A9	NCU05768	4.4	3.9	0.579	<i>poi-2</i> (<i>N. crassa</i>), 0.0
NM1D7	NCU07027	5.7	3.6	0.165	Glycogen phosphorylase (<i>A. fumigatus</i>), 0.0
NM1A6	NCU07784	6.3 (8.0)	2.7 (13)	0.165	Conserved hypothetical protein (<i>M. grisea</i>), e-115
SP4C6	NCU00515	2.1 (ND)	1.5 (ND)	0.124	RING-finger-like domain (cd00162.2), 1e-07
SC3G2	NCU08487	2.6 (1.8)	1.4 (1.3)	0.124	Conserved hypothetical protein (<i>M. grisea</i>), 6e-36
NM2E10	NCU08791	5.5 (1.6)	3.8 (1.8)	0.120	Catalase-1 (<i>N. crassa</i>), 0.0
SM2G3	NCU08332	5.6 (1.7)	4.0 (3.5)	0.108	Woronin body major protein (<i>A. nidulans</i>), 3e-48
NP3E3	NCU08055	2.8 (1.7)	1.4 (1.3)	0.046	Basic-leucine zipper transcription factor (<i>N. crassa</i>), 0.0
NC1H4	NCU08055	2.9	1.3	0.046	Basic-leucine zipper transcription factor (<i>N. crassa</i>), 0.0
NP6D8	NCU02109	2.3 (1.3)	1.2 (0.6)	0.046	UTP-glucose-1-phosphate uridylyltransferase, 1e-49
Class II					
SP1D12	NCU05789	1.0 ^b (1.1)	4.0 (3.4)	0.010	1,3-β-Glucanase (<i>Streptomyces coelicolor</i>), 2e-32

Values in parentheses represent ratio quantitated by phosphorimager analysis of Northern blots. ND, no signal detected in Northern blot analysis.

^a One-way ANOVA *P*-value in comparison between affects of *mak-2* and *pp-1*.

^b *t*-test *P*-value that is not significantly different from 1.0 (not distinguishable from wild type).

^c See Figure 4 for Northern blot.

domains contributes to unique roles of the protein. It will be of interest to test the phenotypes of point mutations that independently eliminate the function of each DNA-binding domain. A conserved proline in the homeodomain region at amino acid position 101 of PP-1 fits the consensus for a MAP kinase-binding site, suggesting a possible interaction between MAK-2 and PP-1.

The *nrc-1* gene, encoding a homolog of the yeast *Ste11* MAPKK kinase, has been characterized (KOTHE and FREE 1998). The *nrc-1* null mutants displayed an abnormal morphology of the hyphae resembling the budding growth observed during conidiophore development. This suggests that the derepressed conidiation observed in *mak-2* mutants might be extreme in the *nrc-1* mutant.

TABLE 4

Genes with elevated expression in *pp-1* and *mak-2* mutants in microarray and Northern blot experiments

EST	Predicted ORF	Average fold change		P-value ^a	BLAST match (organism) <i>e</i> -value
		$\Delta pp-1$ /WT	$\Delta mak-2$ /WT		
Class III					
SM2B5	NCU03171.1	1.9 (2.1)	2.0 (2.0)	0.949	Sexual differentiation process protein <i>isp4</i> (<i>S. pombe</i>), 0.0
SP2A11	NCU05079.1	1.8 (1.9)	2.3 (2.3)	0.381	Similar to peptide transporter PTR2 (<i>C. albicans</i>), 6e-82
CCG-2 1/2	NCU08457.1	2.0 (2.9)	2.0 (3.9)	0.995	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
CCG-2 1/8	NCU08457.1	1.9	2.1	0.331	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
CCG-2 1/4	NCU08457.1	3.0	2.3	0.147	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
CCG-2	NCU08457.1	2.7	1.8	0.034	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
CCG-2	NCU08457.1	2.0	1.3	0.064	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
CCG-2	NCU08457.1	2.5	1.2	0.010	Clock-controlled gene protein 2 (<i>N. crassa</i>), 0.0
SC2F11	NCU07318.1	2.1 (ND)	1.6 (ND)	0.219	Mannitol-1-phosphate dehydrogenase mtID (<i>A. niger</i>), e-132
W10D1	NCU01065.1	3.5 (3.0)	2.4 (3.6)	0.090	Ammonium transporter MEPa (<i>A. nidulans</i>), 0.0
NP4C8	NCU01297.1	2.2 (ND)	1.8 (ND)	0.073	Predicted protein (<i>N. crassa</i>), 0.0
NP4E6	NCU06358.1	2.0 (1.0)	2.9 (3.9)	0.010	Sugar transporter Mst-1 (<i>A. nidulans</i>), e-113
NP4B12	NCU07523.1	2.1 (ND)	1.5 (ND)	0.036	Probable glucan 1,3- β -glucosidase (<i>Blumeria graminis</i>), 0.0
W07E6	NCU03530.1	2.4 (ND)	1.5 (ND)	0.009	Related to chitinase 3 precursor protein (<i>N. crassa</i>), 6e-44
W06B10	NCU09345.1	2.5 (3.1)	1.3 (2.1)	0.010	Thiamine biosynthesis protein NMT-1 (<i>N. crassa</i>), 0.0
NMT-1	NCU09345.1	2.1	1.1	0.004	Thiamine biosynthesis protein NMT-1 (<i>N. crassa</i>), 0.0
W06E6	NCU06380.1	1.6 (ND)	3.6 (ND)	0.010	Contain a ZIP Zinc transporter domain (pfam0235), 2e-39
NM1C4	NCU02500.1	1.6	2.8	0.034	Clock-controlled pheromone precursor (<i>N. crassa</i>), e-160
NC1H2	NCU02500.1	1.5	4.2	0.002	Clock-controlled pheromone precursor (<i>N. crassa</i>), e-160
CCG-4	NCU02500.1	1.1 (2.8)	2.7 (8.4)	0.004	Clock-controlled pheromone precursor (<i>N. crassa</i>), e-160
Class IV					
W13G8	NCU06249.1	2.0 (ND)	1.2 (ND)	0.010	Related to ser/thr-protein kinase (<i>S. cerevisiae</i>), 1e-76
NP4A9	NCU05888.1	2.1 (ND)	0.7 (ND)	0.001	Coenzyme F420-dependent N5, N10-methylene tetrahydromethanopterin reductase (<i>Azotobacter</i>), e-126
Class V					
NM3H1	NCU08870.1	1.5 (ND)	2.4 (ND)	0.147	Conserved hypothetical protein (<i>M. grisea</i>), 4e-18
NM3H4	NCU02793.1	1.4 (ND)	4.4 (ND)	5.74E-04	Conserved hypothetical protein (<i>M. grisea</i>), e-143

Values in parentheses represent ratio quantitated by phosphorimager analysis of Northern blots. Values in italics indicate *t*-test *P*-values that are not significantly different from 1.0 (not distinguishable from wild type). ND, no signal detected in Northern blot analysis.

^a Nonparametric one-way ANOVA (Welch *t*-test) *P*-value in comparison between affects of *mak-2* and *pp-1*.

In addition, *nrc-1* mutants are female sterile and as a male parent produce “flattened” inviable ascospores (KOTHE and FREE 1998). These common phenotypes suggest that *nrc-1*, *mak-2*, and *pp-1* are part of the same MAP kinase-signaling cascade with *nrc-1* acting upstream of *mak-2* and *pp-1* in regulating sexual and asexual development. The more severe phenotypes of mutations further upstream in the MAP kinase cascade indicate the occurrence of regulatory branch points at each step in the pathway. An additional phenotype for *nrc-1*, *mak-2*, and *pp-1* is a defect in hyphal fusion (PANDEY *et al.* 2004), further suggesting a pathway involving these three regulatory genes.

***mak-2* and *pp-1* affect growth and development:** The *mak-2* and *pp-1* mutants have reduced growth rate on solid medium. Reduced growth rate was also reported for the corresponding MAP kinase mutants in other fungi, including *bmp1* in *Botrytis cinerea*, *chk1* in *Cochliobolus heterostrophus*, *fmk1* in *Fusarium oxysporum*, and *ubc3*

in *Ustilago maydis* (LEV *et al.* 1999; MAYORGA and GOLD 1999; ZHENG *et al.* 2000; DI PIETRO *et al.* 2001). However, reduced growth rate was not noted for other fungal species, such as *M. grisea*, *Colletotrichum lagenarium*, and *Claviceps purpurea* (XU and HAMER 1996; TAKANO *et al.* 2000; MEY *et al.* 2002). Interestingly, the function of most *ste12* homologs in other filamentous fungi, such as *MST12* in *M. grisea*, *cph1* in *Candida albicans*, *cls12* in *Candida lusitanae*, *steA* in *A. nidulans*, *stlA* in *Penicillium marneffeii*, and *ste12 a* and *stex* in *Cryptococcus neoformans*, was not required for vegetative growth but was restricted to sexual development and processes involved in pathogenicity (LIU *et al.* 1994; CHANG *et al.* 2000, 2001; VALLIM *et al.* 2000; YOUNG *et al.* 2000; BORNEMAN *et al.* 2001; PARK *et al.* 2002). Thus, the influence of this conserved MAP kinase-signaling pathway on vegetative growth is a species-specific phenomenon.

Functional differences of this MAP kinase pathway also exist in filamentous fungi with respect to asexual

sporulation. The *fus3/kss1* and *ste12* homologs were found to be dispensable for conidiation in *M. grisea* (*PMK1*, *MST12*), *C. purpurea* (*cpmk1*), *B. cinereus* (*bmp1*), *F. oxysporum* (*fmk1*), *A. nidulans* (*steA*), and *P. marneffei* (*stlA*) (XU and HAMER 1996; VALLIM *et al.* 2000; ZHENG *et al.* 2000; BORNEMAN *et al.* 2001; DI PIETRO *et al.* 2001; MEY *et al.* 2002; PARK *et al.* 2002). However, *chk1*-deletion mutants of *C. heterostrophus* have poorly developed aerial hyphae and fail to produce conidia (LEV *et al.* 1999). Similarly, conidiation is “significantly reduced” in *C. lagenarium* *cmk1*-deletion mutants (TAKANO *et al.* 2000). Interestingly, *N. crassa* *mak-2* and *pp-1* null mutants have stunted aerial hyphae, but produce a yield of conidia similar to that of the wild type. These observations indicate that the activities of the *mak-2* and *pp-1* genes are essential for development of aerial hyphae and inhibitory to conidial morphogenesis. Additionally, since the derepression of conidiation in *mak-2* mutants was more severe than that in the *pp-1* mutant (but less severe than that in adenylate cyclase (*cr-1*) mutants (IVEY *et al.* 2002)), it is likely that MAK-2 negatively regulates conidial development through factors in addition to PP-1.

***mak-2* and *pp-1*-regulated genes:** We used subtractive enrichment cloning and microarray analysis to identify genes that are regulated by *mak-2* and *pp-1* (*mkr* genes) to learn how this pathway regulates sexual and asexual development and to identify other processes regulated by this pathway, such as production of secondary metabolites. Because the major phenotypes observed in the mutants were an absence of protoperithecia and aerial hyphae, we expected to identify genes whose expression is specific to these tissue types.

Most genes requiring *mak-2* for their expression also required *pp-1*, supporting the view that activation of genes by the MAK-2 kinase pathway involves PP-1. These class I genes represent a variety of cellular functions but, as expected, include genes expressed during protoperithecia formation. These include *poi-2*, a gene identified as abundantly expressed in perithecia-containing cultures (NELSON *et al.* 1997), and the *mkr-3* gene identified by subtractive enrichment in this study.

The class III genes appear to be downregulated by *mak-2* and *pp-1*. However, class IV and V genes were repressed by either MAK-2 or PP-1 but not by both, potentially indicating the existence of other factors in the MAK-2 kinase pathway for its repression function. This hypothesis might explain the observation that the depression of conidiation in the *mak-2* mutant is more severe than that in the *pp-1* mutant in submerged culture.

Several other *N. crassa* mutants that show similar or overlapping phenotypes with *mak-2* and *pp-1* mutants have been described (PERKINS *et al.* 2001). It is possible that some of these genes are components that are involved in the *mak-2* pathway in *N. crassa*. For example, *asm-1* (*ascospore maturation-1*, NCU01414.1) is a regulatory gene that plays a key role in sexual development and contributes to asexual development as well (ARA-

MAYO and METZENBERG 1996; ARAMAYO *et al.* 1996). Deletion mutants of *asm-1* show phenotypes including stunted aerial hyphae, slow conidial germination, absence of protoperithecia, and ascospore lethality. The ASM-1 protein contains a potential MAP kinase-binding motif (amino acid 289) and several potential serine/threonine phosphorylation sites (data not shown). ASM-1 could be another downstream target of MAK-2.

MAP kinase and the cAMP-signaling pathways: The *N. crassa* *cr-1* gene encodes adenylate cyclase, which regulates the intracellular cyclic AMP (cAMP) level and is the first key component in the PKA pathway in *N. crassa* (IVEY *et al.* 2002). The *cr-1* null mutants share phenotypes with *mak-2* and *pp-1*, including slow growth, lack of aerial hyphae, and dense premature conidiation (KAYS *et al.* 2000; IVEY *et al.* 2002; YANG *et al.* 2002), suggesting the potential for cross talk between the cAMP and MAP kinase-signaling pathways. Different patterns of cross talk between the MAP kinase and the PKA-signaling pathways have been discovered in several fungi (LENGELER *et al.* 2000). However, since the *cr-1* mutants can form protoperithecia and produce viable ascospores after crossing, it is obvious that any cross talk affects only asexual development. Additionally, the vegetative growth defects of *cr-1* null mutants are more severe than those observed for *mak-2* and *pp-1*, and exogenous cAMP does not restore the *mak-2* and *pp-1* aerial hyphae defects as it does for *cr-1* mutants. This indicates that the PKA pathway may act upstream of the MAP kinase pathway to regulate production of aerial hyphae and inhibit premature conidiophore development (Figure 6). Furthermore, three G α proteins and one G β protein, GNA-1, GNA-2, GNA-3, and GNB-1, have been identified in *N. crassa* and shown to be positive regulators of adenylate cyclase (KAYS *et al.* 2000; IVEY *et al.* 2002; YANG *et al.* 2002). Mutations in these G proteins cause defects in vegetative growth, aerial hyphae formation, conidiation, and female fertility, which are partially shared with the *mak-2* and *pp-1* mutants. These G proteins may be indirect regulators of asexual development through their effects on cAMP levels. Additionally, the *gna-1* and *cr-1* double mutants have more severe defects in vegetative and aerial hyphae growth than the single mutants, and, unlike the *cr-1* mutant, the *gna-1* and *gna-1*; *cr-1* double mutants are refractory to rescue by exogenous cAMP (IVEY *et al.* 2002). This indicates that in addition to the known role of *gna-1* in regulating the PKA pathway, the G proteins also regulate asexual development through another pathway, which might also involve the *mak-2* MAP kinase pathway. Furthermore, G proteins also play critical roles in female fertility (KAYS *et al.* 2000; IVEY *et al.* 2002; YANG *et al.* 2002), although the defects are less severe than those for *mak-2* or *pp-1* mutants. Conceivably, these G proteins might have a direct influence on the *mak-2* MAP kinase pathway's role in sexual development, analogous to that of *GPA1* in the *S. cerevisiae* *FUS3*

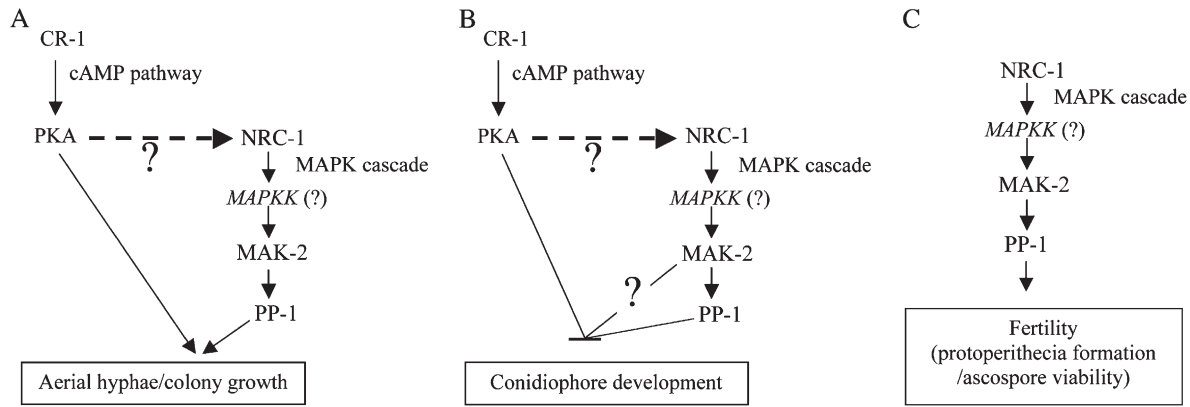


FIGURE 6.—Model for MAP kinase pathway regulation of gene expression and development. (A) The cAMP-signaling pathway may regulate the *mak-2*-related MAP kinase pathway to control aerial development or the two pathways may act independently. (B) Conidiophore development is repressed by the cAMP pathway directly or by its effect on the MAK-2 pathway. On the basis of microarray and enhanced conidiation of the mutants, MAK-2 exerts a stronger effect than PP-1 and MAK-2 may act through PP-1 and another (?) factor to repress conidiation. (C) Protoperithecial development and ascospore lethality depend on the MAK-2 pathway and is independent of cAMP signaling.

MAP kinase pathway (GUSTIN *et al.* 1998). Thus, there are several ways in which the MAP kinase pathway may regulate development by acting downstream of cAMP and G-protein signaling (Figure 6).

Regulation of plant-association genes: Plant cell-wall-degrading enzymes and secondary metabolites are important virulence factors in pathogenesis. In nature, *N. crassa* produces perithecia embedded within colonized plant tissue; thus, production of at least some plant cell-wall-degrading enzymes may be associated with perithecia production and may be under control of the MAP kinase pathway. The *KSSI* MAP kinase pathway positively regulates the expression of a polygalacturonase gene, which is involved in invasive growth in *S. cerevisiae* (MADHANI *et al.* 1999). However, in *N. crassa*, expression of two exopolygalacturonase genes (NCU02369.1 and NCU06961.1) was not dependent on *mak-2* or *pp-1* during sexual or asexual development (data not shown). This is consistent with findings for a polygalacturonase of *F. oxysporum* (DI PIETRO *et al.* 2001). However, the class II gene NCU05789.1 and the class III gene NCU07523.1 encode members of apparent 1,3- β -glucanase gene families in *N. crassa*. No clear homologs of these genes are present in *S. cerevisiae*, however, the plant pathogens *M. grisea* and *F. graminearum* each have multiple members of each of these 1,3- β -glucanase families. Cell-wall-degrading enzymes are considered virulence factors in fungus-plant interactions. Interestingly, the class I gene NCU05521.1 encodes a close homolog of the PATH531 protein from *M. grisea* that is annotated as being required for plant pathogenesis.

Additionally, we observed a gene cluster on chromosome I in *N. crassa*, including three *mkr* genes (*mkr-2*, -3, and -6) and a putative polyketide synthase ORF. Northern blot analysis and RT-PCR results indicate that this secondary metabolite cluster is expressed during sexual

development, particularly during protoperithecia formation. However, the specific function of this *pks* cluster is still unknown. A homologous *pks* cluster in contig 2.2111 in *M. grisea* contains all but 1 of the 11 genes in the *pks* cluster in the *N. crassa* (contig 3.152) with only a few differences in the order and orientations of several genes (Figure 5). This suggests that the conserved genes do form part of a cluster of genes involved in production of a metabolite. It is interesting that no other sequenced organisms contain an orthologous cluster, including *Podospora anserina* (<http://podospora.igmors.u-psud.fr>), a fungus much more closely related to *N. crassa* than is *M. grisea*. Interestingly, *P. anserina* is not a plant-associated fungus. Although *N. crassa* is known to colonize only dead plant material, this polyketide cluster may play a role in promoting plant association in *N. crassa* and *M. grisea*. Another attractive possibility is that such a metabolite may serve as a signal-regulating intrahyphal communication. It has been hypothesized (PANDEY *et al.* 2004) that such a diffusible compound may help regulate hyphal fusion, a phenomenon absent in *mak-2* and *pp-1* mutants. Additionally, the activity of these compounds might be important for protoperithecium morphogenesis.

We conclude that the role of the *mak-2/pp-1* pathway in both sexual and asexual development, and in regulating plant cell-wall-degrading enzymes, secondary metabolites, and homologs of plant pathogenicity genes, is consistent with the dual regulation of the mating process and pathogenicity/invasive growth observed in many fungal pathogens. Thus, analysis of this MAP kinase pathway is likely to provide an important perspective on the evolution of a major regulatory pathway governing fungal pathogenesis. Although we do not know if the ancestor of *N. crassa* was saprophytic or parasitic, *N. crassa* does possess the basic gene content thought to

be essential for plant pathogenesis. We hypothesize that, in general, plant pathogenesis could have evolved through modification of the existing regulatory pathways and gene content found in saprophytic progenitor fungi similar to *Neurospora*.

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