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).

Sequence data from this article have been deposited with the GenBank Data Libraries under accession nos. AF348490 (mak-2) and AY027529 (pp-1).

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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 *PMKI* 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 Borovick 2004) and additional roles in protoperitheciun function (Kim and Borovick 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 divergence between *MST12* and *steA*.

We characterized *N. crassa* homologs of *S. cerevisiae FUS3/KSS1* STE12 genes, called *mak-2* (mitogen activated kinase-2) and *pp-1* (protoperitheciun-1), respectively. Both genes are essential for protoperithecia for-
mation and contribute to maintaining normal filamentous growth and development of aerial hyphae. Several downstream targets 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-ORS6a (FGSC 4200), and σ-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 DeSerres 1970). For RNA extraction, conidia were collected in sterile water from flasks with VM solid medium after 7 days of incubation at 34°C and filtered through Miracloth (Calbiochem, La Jolla, CA). Cultures were inoculated with 10^6 conidia/ml and grown in flasks at 34°C at 250 rpm. Mycelia were harvested by filtration onto filter paper in a Bucher funnel. Mycelia from cellophane overlays on VM and SC plates were collected after 7 days of incubation at 34°C or 25°C, 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 (Perrins 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 × 10^5 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 N. PMK1, X17D4, X3B2, G21F5, and G21G11. The complete sequence for microarray experiments, to reduce the variation arising from the gene was determined and we named the gene mak-2. 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 (Orrbach 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 (protoperithecia-1) because the phenotype of the corresponding mutant strain resembled the previously described pp mutant phenotype (Perrins 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 EcoRI-HindIII hph cassette was cloned into the EcoRI-HindIII polynucleotid sites of pCB1532 to create pBP27. Next, the 2.2-kb BamHI-BamHI fragment containing the mak-2 downstream flanking region was ligated into the BamHI site of pBP27. The 2-kb XhoI-XhoI upstream flanking region of mak-2 was PCR amplified from plasmid pBP30 with T7 and MAK1 (containing an XbaI site) primers and ligated into the XbaI site to produce pBP-KOMAK2. Before transformation into N. crassa 74-ORS6a, pBP-KOMAK2 was linearized with NotI. Hygromycin-resistant transformants were isolated and screened by PCR using primers PDL37 and PDL38. Isolates of each mak-2 knockout mutant were purified to homokaryon 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 EcoRI-HindIII 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 sulfonylurea resistance (100 μg/ml chlorimuron ethyl). Then the sulfonylurea-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 HpaI-HpaI hph cassette from plasmid pCB1004 was
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<th>Sequences</th>
<th>Strains</th>
<th>Genotype/comment</th>
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<tbody>
<tr>
<td>PDL21</td>
<td>5'-GTCGATGTGCCTCAACTTTGCAAC-3'</td>
<td>74-OR23-IVA</td>
<td>Wild-type strain, mat A (FGSC 2489)</td>
</tr>
<tr>
<td>PDL22</td>
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<td>74-ORS6a</td>
<td>Wild-type strain, mat a (FGSC 4200)</td>
</tr>
<tr>
<td>actin1</td>
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<td>PBM5</td>
<td>Δmak-2 A, constructed in 74-ORS6a</td>
</tr>
<tr>
<td>actin3</td>
<td>5'-GGTCCATGAGAATGAGCTTCTCAATCTG-3'</td>
<td>PBMP5-1</td>
<td>Δmak-2 A, F1 progeny of PBM5 cross with 74-OR23-IVA (FGSC 9553)</td>
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<tr>
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<td>PBM49</td>
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<tr>
<td>STE-1</td>
<td>5'-GTCGATGTGAGAATGAGCTTCTCAATCTG-3'</td>
<td>DL14</td>
<td>Δpp-1 A, constructed in 74-OR23-IVA (FGSC 9555)</td>
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<td>FGSC 4345</td>
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<th>Strains</th>
<th>Genotype/comment</th>
</tr>
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<td>PDL37</td>
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<td>mak-2 gene replacement plasmid</td>
</tr>
<tr>
<td>PDL38</td>
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<td>pBP-KOSTE12</td>
<td>pp-1 gene replacement plasmid</td>
</tr>
<tr>
<td>actin1</td>
<td>5'-GTCGATGTGAGAATGAGCTTCTCAATCTG-3'</td>
<td>pDL3</td>
<td>mak-2 gene complementation plasmid</td>
</tr>
<tr>
<td>MTH-1</td>
<td>5'-GTCGATGTGAGAATGAGCTTCTCAATCTG-3'</td>
<td>pBP15</td>
<td>pBluescript SK- vector with an hph cassette in the EcoRV site</td>
</tr>
<tr>
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<td>pBP26</td>
<td>pCB1004 with a 2.5-kb BglII fragment of cosmid G14H8 in the BamHI site</td>
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<td>pCB1004 with an EcoRI-HindIII hph cassette</td>
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<td>pBP29</td>
<td>pCB1004 with a 2-kb XhoI upstream flanking region of mak-2</td>
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<tr>
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<td>pMB1</td>
<td>pCB1004 with a 7.5-kb BamHI fragment of cosmid G14H8 in the BamHI site</td>
</tr>
<tr>
<td>STE-3KO</td>
<td>5'-GTCGATGTGAGAATGAGCTTCTCAATCTG-3'</td>
<td>pMB2</td>
<td>pCB1004 with a 2.2-kb BamHI downstream flanking region of mak-2</td>
</tr>
</tbody>
</table>

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 EcoRI fragment containing the downstream flanking region was cloned into the EcoRI site of pBP15. The 1.6-kb KpnI-HindIII upstream flanking region of pp-1 was obtained from cosmid X17D4 and cloned into the KpnI-HindIII site of pBP15 to create plasmid pBP-KOSTE12 plasmid. Before transformation into N. crassa, pBP-KOSTE12 was digested with NsoI and KpnI 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 (PolyATrace mRNA
Isolation Systems, Promega, Madison, WI) from ~1.5 mg of total RNA, and 1 μg of poly(A)^+ RNA was used to synthesize cDNA (cDNA Synthesis Kit, Amersham Pharmacia Biotech, Piscataway, NJ). cDNAs were digested with RsaI 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 were then normalized by the per spot and per chip intensity, and this biotinylated cDNA was incorporated into the cDNA from the wild type. 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 EcoRII digest. A library of *Escherichia coli* 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 (Cy5 and Cy3) labeling system, which allows simultaneous comparison of gene expression patterns, was used (DeRisi et al., 1997). cDNA samples of wild type (74-OR23-1VA), Δpp-1 (DL14), or Δmak-2 (PPBM5-1) 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 (Gensphere, Hatfield, PA) as described previously (Xie et al., 2004). Fluorescence images were acquired using an Affymetrix 426 array scanner (Affymetrix, Santa Clara, CA) and then imported into ScanAlyze (http://vana.lnl.gov/EisenSoftware.htm) to generate raw signal intensities for each spot. The *N. crassa* microarray data files are available at http://plpak2linux.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 (Δmak-2 vs. wild type and Δpp-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 Benjamini 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 cosmids 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 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 Δmak-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 Δmak-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 Δmak-2 mutants (Figure 1C, right). The PBMP5 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 PBMP5 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-HinIII 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 was 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 Δmak-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 Δpp-1 knockout mutants. (C) Southern analysis of Δmak-2 knockout mutant strains PBMP5, PBMP7, and PBMP49. Genomic DNAs were digested with BamHI (B) or KpnI (K) yielding fragments of the indicated sizes (kb). (D) Southern analysis of the Δpp-1 knockout mutant strain DL14. Genomic DNAs were digested with XhoI (X) or KpnI (K) to yield fragments of the indicated sizes.
Figure 3.—Aerial growth of strains with and without 2 mM cAMP for wild-type, Δpp-1, Δmak-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 Δmak-2 and Δpp-1 mutants were able to fertilize protoperithecia formed by the wild type. Protoperithecia from those crosses developed into mature perithecia with 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 Δmak-2 and Δpp-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 Δmak-2 or Δpp-1 (Figure 3). The cr-1 mutant also produces conidia prematurely in liquid cultures. We noted that when grown in SC liquid medium, the Δmak-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 Δpp-1 mutant began to conidiate ~ 24 hr after inoculation (Figure 2).
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 Δmak-2 mutant, reduced aerial hyphae and female sterility, can be easily detected on SC solid medium, both the wild-type strain and the Δmak-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, Δmak-2, and Δpp-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 Δpp-1 mutant vs. the wild-type strain and three of which were replicates of the cDNA from the Δmak-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

### TABLE 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCU no.</th>
<th>Contig</th>
<th>Expression pattern</th>
<th>BLAST match (organism), e-value</th>
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<tbody>
<tr>
<td>mkr1</td>
<td>NCU08907.1</td>
<td>3.557</td>
<td>Unspecific</td>
<td>Related to blastomyces yeast phase-specific protein (Ajellomyces dermatitidis), 2e-10</td>
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<tr>
<td>mkr2</td>
<td>NCU02923.1</td>
<td>3.152</td>
<td>Protoperithecia specific</td>
<td>Putative short chain dehydrogenase (S. probe), 3e-12</td>
</tr>
<tr>
<td>mkr3</td>
<td>NCU02930.1</td>
<td>3.152</td>
<td>Protoperithecia specific</td>
<td>Putative pyridoxal reductase (S. probe), 7e67</td>
</tr>
<tr>
<td>mkr4</td>
<td>NCU06597.1</td>
<td>3.882</td>
<td>Unspecific</td>
<td>Unknown</td>
</tr>
<tr>
<td>mkr5</td>
<td>NCU07449.1</td>
<td>3.434</td>
<td>Aerial hyphae specific</td>
<td>Unknown</td>
</tr>
<tr>
<td>mkr6</td>
<td>NCU02918.1</td>
<td>3.152</td>
<td>Protoperithecia specific</td>
<td>C terminus similar to binA gene product (A. nidulans), 5e-16</td>
</tr>
</tbody>
</table>

* ORF ID numbers assigned by the Broad Institute (Galagan et al. 2003).
* Contig ID of assembly three of the N. crassa genome (Galagan et al. 2003).
* Based on Northern analysis (Figure 4A; data not shown).
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 (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, mak-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 phosphoimagery 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 Cys/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
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 (Köthe 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>
<thead>
<tr>
<th>EST</th>
<th>Predicted ORF</th>
<th>Average fold change</th>
<th>BLAST match, e-value</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>NM6A4</td>
<td>NCU04931</td>
<td>2.9 (2.3) 2.8 (3.7)</td>
<td>0.838 Conserved hypothetical protein (N. crassa), 0.0</td>
</tr>
<tr>
<td>NP6A12</td>
<td>NCU04931</td>
<td>2.7 4.0</td>
<td>0.046 Conserved hypothetical protein (N. crassa), 0.0</td>
</tr>
<tr>
<td>NP2E11</td>
<td>NCU05236</td>
<td>6.3 7.0</td>
<td>0.738 Predicted protein (N. crassa), 1e-63</td>
</tr>
<tr>
<td>NM3D7</td>
<td>NCU05236</td>
<td>5.8 (33) 8.3 (50)</td>
<td>0.354 Predicted protein (N. crassa), 1e-63</td>
</tr>
<tr>
<td>NM1H4</td>
<td>(chimeric) NCU08812</td>
<td>4.4 (ND) 5.9 (ND)</td>
<td>0.592 Conserved hypothetical protein (A. nidulans), 7e-56</td>
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<tr>
<td>SP4A5</td>
<td>NCU07598</td>
<td>2.3 (ND) 1.9 (ND)</td>
<td>0.545 Related to salicylate 1-monooxygenase (Emericella nidulans), 5e-55; related to cysteine desulfurase NFS1, 1e-38</td>
</tr>
<tr>
<td>NM5F1</td>
<td>NCU04416</td>
<td>2.0 (4.1) 3.1 (3.3)</td>
<td>0.368 Conserved hypothetical protein (N. crassa), 0.0</td>
</tr>
<tr>
<td>NM5D2</td>
<td>NCU07166; (chimeric) NCU04533</td>
<td>6.1 (ND) 4.7 (ND)</td>
<td>0.299 DNA-dependent RNA polymerase I (S. cerevisiae), 1e-16; Ca-dependent cell adhesion molecule-1 (Dictyostelium), 9e-11</td>
</tr>
<tr>
<td>NM7F7</td>
<td>NCU02988</td>
<td>6.7 (7.6) 3.9 (9.9)</td>
<td>0.298 Related to ubiquinone/menaquinone biosynthesis methyltransferase (COG2261.1, UbiE), 5e-05</td>
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<tr>
<td>NM8A5</td>
<td>NCU02259</td>
<td>6.1 (7.4) 3.4 (6.7)</td>
<td>0.294 Related to LST7 amino acid permease Golgi transport protein (S. cerevisiae), 2e-09</td>
</tr>
<tr>
<td>NM5C5</td>
<td>NCU04603</td>
<td>4.6 (6.9) 3.1 (12)</td>
<td>0.294 Riboflavin aldehyde-forming enzyme (Agaricus), 2e-08</td>
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<td>W10D8</td>
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<td>5.8 (16) 3.8 (14)</td>
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<td>NM3H7</td>
<td>NCU08691</td>
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<tr>
<td>NM4G7</td>
<td>NCU01724</td>
<td>7.4 (3.8) 5.0 (4.9)</td>
<td>0.165 Related to Na+/H+-exchange protein (S. cerevisiae), 2e-66</td>
</tr>
<tr>
<td>NM8A11</td>
<td>NCU02532</td>
<td>4.2 (ND) 2.7 (ND)</td>
<td>0.165 Probable ubiquitin-activating enzyme (N. crassa), 0.0</td>
</tr>
<tr>
<td>SM3D2</td>
<td>NCU02930</td>
<td>11.9 4.9</td>
<td>0.165 mkr-3, pyridoxal reductase (S. pombe), 4e-67</td>
</tr>
<tr>
<td>NM1A10</td>
<td>NCU04088</td>
<td>7.4 (ND) 4.1 (ND)</td>
<td>0.165 Cell wall surface anchor family protein (Streptococcus), 7e-10</td>
</tr>
<tr>
<td>NM7F6</td>
<td>NCU05521</td>
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<td>0.165 Pathogenicity protein (PATH531) mRNA (M. grisea), 9e-64</td>
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<td>SP4D12</td>
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<td>5.4 2.5</td>
<td>0.165 poi-2 (N. crassa), 0.0</td>
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<td>SM1B11</td>
<td>NCU05768</td>
<td>7.9 3.3</td>
<td>0.165 poi-2 (N. crassa), 0.0</td>
</tr>
<tr>
<td>NM1A11</td>
<td>NCU05768</td>
<td>7.5 (7.3) 6.0 (9.0)</td>
<td>0.165 poi-2 (N. crassa), 0.0</td>
</tr>
<tr>
<td>NM1F7</td>
<td>NCU05768</td>
<td>6.3 3.5</td>
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<tr>
<td>SP4F9</td>
<td>NCU05768</td>
<td>3.2 1.4</td>
<td>0.010 poi-2 (N. crassa), 0.0</td>
</tr>
<tr>
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<td>0.579 poi-2 (N. crassa), 0.0</td>
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<tr>
<td>NM1D7</td>
<td>NCU07027</td>
<td>5.7 3.6</td>
<td>0.165 Glycogen phosphorylase (A. fumigatus), 0.0</td>
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<td>NM1A6</td>
<td>NCU07784</td>
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<tr>
<td>SP4C6</td>
<td>NCU00515</td>
<td>2.1 (ND) 1.5 (ND)</td>
<td>0.124 RING-finger-like domain (cd00162.2), 1e-07</td>
</tr>
<tr>
<td>SC3G2</td>
<td>NCU08847</td>
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<td>0.124 Conserved hypothetical protein (M. grisea), 6e-36</td>
</tr>
<tr>
<td>NM2E10</td>
<td>NCU08791</td>
<td>5.5 (1.6) 3.8 (1.8)</td>
<td>0.120 Catalase-1 (N. crassa), 0.0</td>
</tr>
<tr>
<td>SM2G3</td>
<td>NCU08332</td>
<td>5.6 (1.7) 4.0 (3.5)</td>
<td>0.108 Woronin body major protein (A. nidulans), 3e-48</td>
</tr>
<tr>
<td>NP3E3</td>
<td>NCU08055</td>
<td>2.8 (1.7) 1.4 (1.3)</td>
<td>0.046 Basic-leucine zipper transcription factor (N. crassa), 0.0</td>
</tr>
<tr>
<td>NC1H4</td>
<td>NCU08055</td>
<td>2.9 1.3</td>
<td>0.046 Basic-leucine zipper transcription factor (N. crassa), 0.0</td>
</tr>
<tr>
<td>NP6D8</td>
<td>NCU02109</td>
<td>2.3 (1.3) 1.2 (0.6)</td>
<td>0.046 UTP-glucose-1-phosphate uridylyltransferase, 1e-49</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP1D12</td>
<td>NCU05789</td>
<td>1.0 1.1 4.0 3.4</td>
<td>0.010 1,3-β-Glucanase (Streptomyces coelicolor), 2e-52</td>
</tr>
</tbody>
</table>

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

`P-value that is not significantly different from 1.0 (not distinguishable from wild type).

See Figure 4 for Northern blot.
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 lusitaniae*, *stelA* in *A. nidulans*, *stla* in *Penicillium marneffei*, and *ste12 a* and *stax* 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 (FMK1, MST12), C. purpurea (cpmk1), B. cinerea (bnp1), 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, chkl-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 inhibit 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 (Aramayo 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. These observations indicate that activation of MAP kinase pathway may act upstream of the MAP kinase pathway to regulate production of aerial hyphae and inhibit premature conidiophore development (Figure 6). Furthermore, three Gα proteins and one Gβ 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
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 KSS1 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 exo-polygalacturonase 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 protoperithecia 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

**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.
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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LITERATURE CITED


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