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Sex-specific gene expression during asexual development of *Neurospora crassa*

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Abstract

The impact of loci that determine sexual identity upon the asexual, dominant stage of fungal life history has been well studied. To investigate their impact, expression differences between strains of different mating type during asexual development were assayed, with RNA sampled from otherwise largely isogenic *mat A* and *mat a* strains of *Neurospora crassa*at early, middle, and late clonal stages of development. We observed significant differences in overall gene expression between mating types across clonal development, especially at late development stages. The expression levels of mating-type genes and pheromone genes were assayed by reverse transcription and quantitative PCR, revealing expression of pheromone and receptor genes instrains of both mating types in all development stages, and revealing that mating type (*mat*) genes were increasingly expressed over the course of asexual development. Interestingly, among differentially expressed genes, the *mat A* genotype more frequently exhibited a higher expression level than *mat a*, and demonstrated greater transcriptional regulatory dynamism. Significant up-regulation of expression was observed for many late light-responsive genes at late asexual development stages. Further investigation of the impact of light and the roles of light response genes in asexual development of both mating types are warranted.

Keywords

mating type; pheromone; transcription; light; conidiation; microarray

1. Introduction

The genetics of sexual identity in most fungi are conferred by mating-type loci that exhibit diversity in size, number, and sequence among different fungal groups (Lee et al., 2010). Within the life cycle of heterothallic fungi, mating occurs when hyphae/conidiospores

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(conidia) from opposite mating-type strains meet at a pre-sexual stage, leading a diploid stage after fusion of nuclei from both mating-types (Esser, 1971). The function of mating-type genes has been intensively studied in fungal models during crossing and sexual development (Glass and Lee, 1992; Saupe et al., 1996; Ferreira et al., 1998; Heitman et al., 2007). However, vegetative stages of the life cycle, encompassing hyphal growth, branching, anastomosis, and asexual sporulation, are generally dominant in fungal life histories.

In general, fungal mating type is not considered to have a significant impact on the growth or phenotypic characteristics of individuals (Coppin et al., 1993; Brasier, 1999), a finding supported by research conducted on diverse fungal species (Dudzinski et al., 1993; Ahmed et al., 1996; Bardin et al., 1997). Nevertheless, an association between mating type and fungal pathogenicity has been demonstrated Kolmer and Ellingboe, 1988; Funnell et al., 2001; Lin et al., 2006). Furthermore, in *Neurospora*, a superiority in perithecial production *of mating-type a* (*mat a*) strains was observed in both intraspecific and interspecific crosses (Dettman et al., 2003). Indeed, genetic differences between mating types relating to the mating loci and their up/downstream regulated pathways have been investigated intensively in *Neurospora*, yeasts, and some other fungi (Heitman et al., 2007), whereas orthogonal studies of spatial differentiation in colony development without consideration of mating type have been performed in *Aspergillus niger* and *N. crassa* (Levin et al., 2007; Kasuga and Glass, 2008; Greenwald et al., 2010). Recently, regulation of mating types on their target genes were investigated using genome wide gene expression profiling for heterothallic fungus *Podospora anserina*, and mating-type transcription factors were found to have impact on genes not directly related to mating in *P. anserina* as well as in *Gibberella moniliformis* (Keszthelyi et al., 2007; Bidard et al., 2011). Although genome-wide transcriptional profiling in *N. crassa* has been applied to identify genes expressed in diverse stages of development (Bell-Pedersen et al., 1996; Nelson et al., 1997; Zhu et al., 2001; Kasuga and Glass, 2008; Greenwald et al., 2010), it has not been applied to ascertain life history differences between mating types. Further characterization of gene expression associated with mating type in *N. crassa* will be of great interests for future studies of the basic phenomena of life such as mating, asexual and sexual reproduction, and mitotic recombination in fungi.

In the heterothallic model fungus *N. crassa*, a bipolar mating system is conferred by the idiomorphic *mat* locus, which encodes *mat a* and *mat A* in opposite mating types. The *mat a-1* gene, encoding a single HMG box protein called MAT a-1, has been identified as the major mating regulator in *mat a* strains (Chang and Staben, 1994), although the additional transcribed ORF *mat a-2* is also identified in this region (Pöggeler and Kück, 2000). The genes of the *mat A* idiomorph encode three proteins: MAT A-1, A-2 and A-3 which are characterized by MAT - HMG (Martin et al., 2010), PPF (Kanematsu et al., 2007), and MATA-HMG domains (Ferreira et al., 1996, 1998). Differential gene regulation for the two mating types should be traceable to differential regulation by genes at the *mat* locus, which, in trans, is known to regulate the expression of presumably mating-type specific pheromone precursors and receptors during the process of mating (Pöggeler and Kück, 2001; Bobrowicz et al., 2002; Kim et al., 200; Kim and Borkovich, 2004, 2006; Pöggeler, 2011). Knockout strains of *mat* loci also show no morphological differences from wild-type in *N. crassa* during vegetative growth (Ferreira et al., 1998). Nevertheless, pheromone genes are maintained in the genomes of true homothallic fungi like *Neurospora africana*, *Sordaria macrospora*, and *Anixiella sublineolata*, and are expressed in the asexual life cycle of *N. africana*, *Gibberella zeae*, and *S. macrospora*, suggesting an alternate or pleiotropic function in vegetative development (Kim et al., 2002; Pöggeler et al., 2006; Lee et al., 2008). Although *mat* genes are undergoing genetic decay in some homothallic species, including *N. africana*, the ORF of *mat A-1* was intact in all these species (Wik et al., 2008). Perhaps in

part due to poorly understood pleiotropic functions, previous studies have revealed conflicting results with regard to the expression of pheromone precursors in strains of different mating types. Pheromone precursor gene *mfa-1* has been attributed specific function only in *mat a*, yet it can be detected at a low expression level in *mat A* tissues (Kim et al., 2002).

The functions of *N. crassa* mating type proteins and pheromone precursors and receptors in the mating processes are well studied. However, their functions during pre-mating asexual development are not clear. In the recent studies of *Podospora anserina* and *Sordaria macrospora*, mating type specific expression was observed for genes with diverse function, includingmetabolism, information pathways, transport, and developmental processes (Bidard et al., 2011; Klix et al., 2010). However, these studies focused on crossing and sexual development, and no core genes active in asexual development, such as the cell division cycle genes (*cdc*), conidiation genes (*con*), and heat shock protein genes (*hsp*), were found differently expressed between mating types. During asexual development, these genes are of critical function, and regulation of many these genes, including clock-controlled genes (*ccg*) and *con* genes, has not been well understood. In most organisms, circadian oscillators regulate the rhythmic expression of *ccgs*, and the two best characterized *ccgs* in *Neurospora* are*ccg-1* and*ccg-2*, known as morning-specific genes. Whilethe precise function of *ccg-1*, which is conserved among filamentous fungi and can be induced by heat shock, is elusive, the gene *ccg-2* encodes a secreted hydrophobic protein belonging to the hydrophobins, which coat the outer cell wall of fungi and maintain the cell-surface hydrophobicity for air dispersal of mature conidiospores (Bell-Pedersen et al., 1992; Vitalini et al., 2006). The production and release of conidiospores in fungi is also subject to the circadian clock, and daily rhythms in spore development and spore discharge are common in fungi (Bell-Pedersen et al., 1996). At least four *con* genes, including *con-6*, *con-8*, *con-10*, and *con-11*, are known to be expressed during development of three types of spores in *N. crassa* (Sachs and Yanofsky, 1991; Springer, 1993). Nevertheless, disruption of these genes does not cause a discernible phenotype in spore morphology, abundance of spores, conidial germination efficiency, nor ability to function as either parent in sexual crosses (Springer and Yanofsky, 1992; Springer, 1993).

In this study, we investigated the global expression differences between largely isogenic strains of different mating type. Mating-type specific expression of genes was quantified using genomic microarrays. The minimal differences in genetic background between highly isogenic mating types provided a straightforward system for investigating transcriptomic shifts of metabolic and regulatory function during morphological development. To maintain a controlled environment for investigating asexual development, the light-induced internal oscillator of *N. crassa* was repressed by a long treatment of constant light, and to avoid temperature-retained clock, a constant temperature was maintained through the experiment. Even under such controlled constant-light conditions, nominally light-responsive genes continue to play a central role in fungal development, so we investigated the behavior of light responsive genes (Chen et al., 2009) for clonal development under a condition of constant light. Furthermore, we performed reverse transcription and quantitative polymerase chain reaction (RT-qPCR) to determine, for the first time, expression of *mat* genes, the pheromone precursor genes *ccg-4* and *mfa-1*, and the receptor genes *pre-1 and pre-2* at different stages of clonal development in *N. crassa*.

2. Experimental procedures

2.1. Strains and conditions

N. crassa strains FGSC 4200 (*mat a*) and FGSC 2489 (*mat A*) were obtained from the Fungal Genetics Stock Center (Kansas City, MO). FGSC 4200 was derived from a long

series of recurrent backcrosses to strain FGSC 2489, and is generally regarded as highly isogenic to the latter (Mylyk et al., 1974; Newmeyer et al., 1987; Perkins, 2004; McCluskey et al., 2010). The strains were grown on Bird Medium (Metzenberg, 2004) covered by a cellophane membrane (Fisher Scientific) at 26°C under constant light. Light was provided by Ecolux bulbs (F17T8.SP41-ECO, General Electric Company) amounting to a net intensity of 14μMol/m^2S at the media surface, measured at wavelengths between 400nm to 700nm. Mycelia were harvested from 90 mm Petri dishes at 36 h, 60 h, and 96 h, corresponding to vegetative growth (colony-size 30-50mm), start to middle of conidia production (50-70mm), and post-peak of conidia production (90 mm), respectively. From 96 h to 144 h in Bird Medium, no protoperithecia were formed. After inoculation, fungal tissues (mycelia) that covered the surface of cellophane membrane were collected with razor blade, snap frozen in liquid nitrogen, and stored at -80°C. We compared the overall gene expression patterns of the whole clones, which are composed of vegetative hyphae and asexual reproduction structures, among different time points for strains of both mating types. Our sampled gene expression pools were thus presumably more heterogeneous than in other sampling methods that address gene expression associated with development of specific hyphal morphology. Nevertheless, we demonstrated improved resolution in identifying differentially regulated genes.

2.2. Multi-targeted priming (MTP) design

We used multi-targeted primers (MTPs), degenerate oligonucleotides complementary to mRNAs but not non-coding RNAs, to facilitate selective reverse transcription of mRNA and elimination of contamination by rRNA and tRNA, leading to improved microarray assay sensitivity (Adomas et al., 2010). Adomas et al. (2010) identified an MTP (VWNVNNBDKGGC) that exactly targets 9826 ORFs in *N. crassa* (85%), that additionally showed strong binding (GC) in the 3′ end. It inexactly primes reverse transcription of all other known transcribed genes as well (Adomas et al., 2010 and unpublished data).

2.3. Sample preparation and hybridization microarrays

Total RNA was extracted from homogenized tissue using TRI REAGENT kits (Molecular Research Center) for three to ten biological replicates for the same time point, and was pooled together for next step. Messenger RNA was purified using Oligo(dT) Cellulose Columns (Molecular Research Center) as in Clark et al. (2008). For reverse transcription, we used 2 μg of purified mRNA and 0.25 μg oligo(dT) mixed with 0.25μg Neurospora-specific MTP. The resulting cDNA was labeled reciprocally with cyanine dyes (Townsend and Hartl, 2002) and used for hybridization. To rule out any accidental contamination with tissue or RNA from the opposite mating type, detection of expression of mating loci *mat A* and *mat a* was used, validating the purity of all RNA samples. Twenty-six hybridizations were performed, including dye-swaps originating from independent reverse transcription reactions and some technical replicates where three or more comparisons were made (Fig. 1). Microarrays were composed of 70mer oligonucleotides synthesized by Illumina (San Diego) for 9,826 ORFs identified by the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora) as in Kasuga et al. (2005), robotically printed on CMT gamma-aminopolysilane-coated glass slides (Corning, Corning, NY) at the Yale University Center for Genomics and Proteomics.

2.4. Microarray data acquisition and analysis

Hybridized microarray slides were scanned with a GenePix 4000B (Axon Instruments, Foster City, CA). Spots were located and expression was quantified by the GenePix 4000 software. All spots were verified by eye, and those with unusual morphology or with erratic signal intensity distribution were excluded from all analyses. Raw intensity was normalized as in Townsend (2004). Genes were considered expressed and deemed well-measured when

the median spot foreground exceeded the median background plus two standard deviations of the background intensity. Normalized data were then statistically analyzed using Bayesian Analysis of Gene Expression Levels (BAGEL, Townsend and Hartl, 2002; Townsend, 2004). We additionally performed BAGEL analyses on subsets of the experiment. After normalization and BAGEL analyses, we obtained well-measured gene expression data for 4,491 genes out of 9,826 genes printed on the array (Table I, Supplemental Table S1). Well-measured genes were considered significantly differentially expressed when $P \leq 0.05$.

To quantify the power of the experiment, the gene expression level at which there was a 50% empirical probability of a significant call (GEL_{50}) was estimated by a logistic regression of statistical significance against $\log_2$ fold-change (Townsend, 2004). To ensure that no subset of the data had greater experimental power due to technical differences in microarray quality, we examined the statistical powers for each subset. The GEL_{50} values were largely consistent among the subsets with one exception concerning the 60 to 96h subset (Table I). Thus, differences in numbers of detected genes were mostly biological in origin and not attributable to differences in quality of hybridization or statistical power applied to each subset.

2.5. Real-time quantitative PCR

Reporter oligonucleotides for genes *mat a-1 and mat a-2* were not present on our microarray. Additionally, expression of mating type genes, pheromone precursor *ccg-4*, and receptor *pre-2*, was not detected with the microarray. Accordingly, we measured transcription of these genes and pheromone precursor *mfa-1* and receptor *pre-1* by reverse transcription and quantitative PCR. The function of *mat a-2* has not been well characterized in *Neurospora crassa*, and we did not assess its expression level during asexual development in this study. RT-qPCR analyses were also used to verify microarray results on selected genes. For RT-qPCR analyses, 2μg of mRNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) for each of two biological replicates, which were pooled mRNAs extracted from three to 10 plates under the same culture condition as for the microarray samples, and for three technical replicates with each of two concentrations (50nM and 300nM) of primers. Relative transcript abundance was quantified using the Applied Biosystems 7500 Fast Real-Time PCR System and SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer's recommendations. The ATP citrate lyase and acyl-CoA dehydrogenase, whose expression was invariant in the two *N. crassa* strains in all time points for both mating types of the microarray experiment, were used as endogenous control references for qPCR. The comparative C^t ($^{\Delta\Delta}C^t$) method (Livak and Schmittgen, 2001) was used to calculate transcript levels from triplicates. Real-time PCR normalization and standard error computation followed Applied Biosystems protocols (Table II) and statistical analyses were performed with BAGEL (Townsend, 2004) as well (Table I, Supplemental Table S1).

2.6. Functional and phylogenetic classification of genes with mating type dependent gene expression

The Functional Catalogue (FunCat: http://mips.helmholtz-muenchen.de/proj/funcatDB/) provided groupings of genes according to their cellular or molecular functions (Ruepp et al., 2004). We partitioned genes that showed monotonically increasing or monotonically decreasing patterns in expression for each mating type. We examined p-values for the comparisons of the neighboring stages (i.e., 36 h vs. 60 h and 60 h vs. 96 h) to identify genes showing increasing or decreasing patterns with credibility higher than 95% for each comparison (TableIII). Statistical significance of overrepresentation of gene groups in functional categories relative to the whole genome was calculated by applying the

hypergeometric distribution to the MIPS gene groups (http://mips.gsf.de/proj/funcatDB/help_p-value.html). Further functional analysis of genes and pathways with significantly differential expression was performed using biochemical pathways and annotation from the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa and Goto, 2000).

In this study, genes exhibiting different expression patterns between mating types during clonal development were mapped onto the classification of Kasuga et al. (2009) for their phylogenetic distribution (Table IV). Genes were classified into six mutually exclusive clade-specific groups: Eukaryote/Prokaryote-core, Dikarya-core, Ascomycota-core, Pezizomycotina-specific, *N. crassa-orphans*, and "others". Three threshold values of length-adjusted protein identity were used by Kasuga et al. (2009) to group genes. We used the classification based on the lowest threshold values, which provided us the strictest list of *Neurospora* orphan genes from that study.

2.7. Supporting information and the Neurospora functional genomics microarray database

The complete expression data set is available in the supporting information (Table SI), on the Townsend Lab web site (http://www.yale.edu/townsend/), and in the filamentous fungal gene expression database (FFGED, Zhang and Townsend, 2010). NCU numbers correspond to the second NCU version of *N. crassa* genome annotation, unless otherwise specified. Specific primers designed for the RT-qPCR were provided as well (Supplemental Table S3). The data set is also available at the Gene Expression Omnibus of the National Center for Biotechnology Information (GEO http://www.ncbi.nlm.nih.gov/geo/) as accession GSE26209.

3. Results

3.1. Genome-wide quantified expression of genes associated with asexual development

A total of 4491 genes were well measured during asexual development (Table I), including genes playing key roles in asexual development. Among 3848 genes expressed in *mat A* during asexual development, 86 genes exhibited no detectable expression in any stage in *mat a*. Among 3553 genes expressed in *mat a*, 11 genes were not detected in any stage in *mat A*. Among 4296 genes detected for both mating types from 36 h to 60 h of development, expression of 106 genes was not detected at 96 h stage for at least one mating type. Among 3403 genes substantially expressed in both 60 h and 96 h stages, expression of 58 genes was not detected at 36 h stage for at least one of the mating types. We found 106 out of 4296, 58 out of 3402, 86 out of 3847, and 11 out of 3553 genes only detected in the subsets respectively (Table I). For 36 h, 60 h, and 96 h comparison between mating types, we observed 44, 61 and 469 genes expressed significantly higher in *mat A* strain while 233, 159, and 744 genes expressed significantly higher in *mat a* strain (Table I, Supplemental Table S1).

Genes of special interest in asexual development, including numerous *hsp*, *cdc*, *con*, *ccg* genes, and known transcription factors, showed significant changes of expression level across clonal development. Expression of *ccg-1*, *ccg-2*, and three *con* genes, *con-6*, *con-8*, and *con-10*, was well measured in this study (Table I, Supplemental Table S4). The expression of *ccg-1* was generally similar between mating-types, but a significantly decreased expression during asexual development for *ccg-2* was observed for only *mat a* in both microarray and RT-qPCR experiments. The expression pattern of *con* genes was also similar between mating-types, including down-regulated expression of *con-6* and up-regulated expression of *con-8* across asexual development. Genes of interest were further verified by RT-qPCR for all three time points (Table S2). Among the 17 selected genes, 9 genes, including *hsp101*, *cdc3*, *cdc4a*, *cdc4b*, C2H2 TFs (CRE-repressor, and *fle*), *con-8*,

ccg-1, and *ccg-2*, exhibited corresponding expression patterns via RT-qPCR assay to those estimated from the microarray experiment, but with higher fold-changes. While the microarray result was not statistically significant, the genes encoding transcription factors NCU04179 and NCU03184 both exhibited up-regulation by RT-qPCR. Conidiation-related gene *con-10* exhibited an increasing expression at later development stages for both mating types in the microarray experiment, and our RT-qPCR results agreed with this result except that highest expression for *con-10* was measured at 36 hr for the *mat a* strain. Expression of some genes that were not detected by the microarray experiment in either mating type, like *hsf2*, or that were detected in just one of the mating types, such as *cdc6*, *cdc12*, *tea1*, and the *ste18*-like gene NCU00041, were detected by RT-qPCR. RT-qPCR provides substantial validation of microarray results, as it is a more sensitive assay than a microarray for detection of differences in expression for genes expressed at low levels (Wang et al., 2006), especially in this study, when more biological replicates were assayed performing real time PCR.

3.2. Real-time quantitative PCR for mating type genes, pheromone precursors and receptors

During asexual development, all mating type genes monotonically increased in expression during the course of asexual development (Fig. 2). In the *mat A* strain, the expression of all three *mat A* coding genes, *mat A-1*, *mat A-2*, and *mat A-3*, increased between 36 h and 96 h, and a 250 fold increase was measured for *mat A-2* from 36 h to 96 h. The *mat a-1* (GenBank P36981.2) gene exhibited a steady and linear increase in expression compared to the *A* mating type genes. Pheromone precursors *ccg-4* and *mfa-1* are regulated separately by *mat A-1* and *mat a-1* (Nelson et al., 1997; Bobrowicz et al., 2002; Kim et al., 2002; Kim and Borkovich 2006), and we observed previously reported expression patterns, but also found the pheromones and receptors to be regulated in the other mating-type (Table II). Consistent with previous studies, different levels of expression of pheromone precursors *ccg-4* and *mfa-1*, and the receptors *pre-1 and pre-2*, were observed in all samples from both mating types (Table II). For the *mat A* strain, expression of *ccg-4* is regulated by the *mat A-1* gene products with a circadian rhythm (Bobrowicz et al., 2002). Our experiments demonstrated a strong correlation between the increasing expression patterns of *ccg-4* and*mat A-1*, with a 3-fold increase of *ccg-4* from 36 h to 60 h and a nearly 80-fold increase from 60 h to 96 h in *mat A*. A less than statistically significant increase (about 1.2 to 1.5 in fold change) of *pre-2*, the putative receptor of *ccg-4*, was observed. We detected about a ten-fold increase in expression of *mfa-1*, a pheromone precursor generally considered to be *mat a* specific, at the late development stage of the *mat A* strain. For the gene *pre-1*, the putative receptor of the MFA1 pheromone, a peak at 60 h samples of *mat A* was observed. For the *mat a* strain, expression of *mfa-1* is probably regulated by the *mat a-1* product and by the circadian clock (Bobrowicz et al., 2002). However, transcription of *mfa-1* peaked at 60 h, then dropped significantly, while transcription of *mat a-1* peaked at 96 h. Interestingly, transcription of both *pre-1* and *pre-2* increased during clonal development, peaking at 96 h in the *mat a* strain.

Transcription of *mfa-1* and *pre-1* was also measured with microarray analysis, and neither gene showed a significant change in transcription during the asexual development in either mating type, which was not consistent with our RT-qPCR results. Expression of pheromone precursors *ccg-4* and *mfa-1* was known to be low in vegetative growth and high in conidia (Bobrowicz et al., 2002; Kim et al., 2002; Kim and Borkovich 2006), but expression of these genes was not well-measured in reproduction of conidia using the same microarray (Kasuga and Glass, 2008). As our RT-qPCR results for all pheromone precursors/receptors and mating-type genes were based on more biological replicates, and showed corresponding

expression patterns in that they were all significantly up-regulated during conidiation, we deemed the RT-qPCR measurements of transcription for these genes to be more accurate.

3.3. Up- and down-regulation of gene sets within functional categories of genes

Significant enrichment in different functional categories was observed for genes showing different expression patterns between mating types. The expression of 79 and 64 genes increased significantly, and 66 and 102 genes decreased significantly, across asexual development for *mat A* and *mat a*, respectively. Of those, we found 78, 61, 61, and 95 respectively, in the Functional Category database (Ruepp et al., 2004; Table III). Seventy-eight genes of increasing expression in *mat A* were classified into 25 functional categories as significantly enriched ($P < 0.05$, Table II Table III A), including genes involved in homeostasis of metal ions and meiosis. In contrast, for 61 genes of increasing expression in *mat a*, only three functional categories exhibited significant enrichment. In other words, the number of Funcat categories that showed significant enrichment was much higher for *mat A*, and most of these categories were not shared between mating types. Three categories exhibited significant enrichment in both: channel/pore class transport, oxygen and radical detoxification, and metabolism of alkaloids. For genes with decreasing patterns, 95 genes were classified into 17 functional categories as significantly enriched in *mat a* (Table III B). These included virulence and disease factors and metabolism of the cysteine – aromatic group. However, none of these categories was enriched with down-regulated genes in *mat A*, except for the biosynthesis of threonine. Down-regulated genes in *mat A* were enriched for 12 other functional categories, including mitochondrial and nuclear transport.

3.4. Expression of light response genes during the clonal development

Genes in *N. crassa* that are known to respond to induction by light exhibited a similar pattern of early and late induction, albeit over a much longer period of time, during asexual development under constant light. More than 300 early light responsive genes (ELRGs) and late light responsive genes (LLRGs) are known to change in expression after light induction (Chen et al., 2009). From 126 ELRGs and 157 LLRGs on the *N. crassa* array, expression of 59 ELRGs (Table S5) and 80 LLRGs (Table S6) were detected during asexual development for both mating types (Fig. 3). Among the 59 ELRGs detected, there were 13 genes in the *mat a* genotype and 25 genes in the *mat A* genotype exhibiting significant up-regulation from 36 h to 96 h ($P < .05$). The largest increase in expression was observed for a NonF related protein NCU06603 (2.7 in *mat a* and 3.1 in *mat A*) and for a probable cyanate lyase NCU01258 (1.8 in *mat a* and 2.7 in *mat A*). There were 11 genes in the *mat a* genotype and 12 genes in the *mat A* genotype showing significant down-regulation from 36 h to 96 h ($P < .05$). The largest decrease in expression was of a probable NADPH2 dehydrogenase NCU04452 (1.3 to 2.3-fold) in both mating types. Among the 80 LLRGs detected, there were 52 genes in the *mat a* genotype and 55 genes in *mat A* showing significant up-regulation from 36 h to 96 h ($P < .05$). The largest differences across time points in expression were of *con-8* (16-fold in *mat a* and 19-fold in *mat A*), NCU05490 (7-fold in *mat a* and 16-fold in *mat A*), and NCU00322 (14-fold in *mat a* and 7.5-fold in *mat A*). Changing expression across asexual development in LLRGs was widespread. There were more than 20 LLRGs in both mating types that exhibited up-regulation over 3-fold from 36 h to 96 h, but we found no LLRGs showing significant down-regulation from 36 h to 96 h.

3.5. Phylogenetic depth of genes differentially expressed between mating types in asexual development

Because mating-type genes are fast evolving, it is helpful to the elucidation of their functional interactions with the rest of the genome to examine the phylogenetic depth of genes differentially expressed between mating types. Mating-type impacted expression of genes evolving at diverse rates. Divergent expression patterns for mating types during clonal

development from this study were mapped onto the phylogenetic distribution classification of Kasuga et al. (2009, Table IV). Many differences between the two mating types in gene expression during asexual development did not correspond to fast-evolving, novel *Neurospora*- specific genes. Most expression differences between *mat A* and *mat a* were detected in genes with orthologues present in the core genomes of Eukaryotes and Prokaryotes (47%, 802 out of a total 1705 significant differences observed). A lower frequency of differential expression between *mat A* and *mat a* was observed for Pezizomyceta-specific genes (27%) and *Neurospora* orphan genes (11%).

4. Discussion

We have provided the first genomic characterization of gene expression differences during clonal development within both mating types of *N. crassa*. Gene expression levels for both mating types throughout vegetative development were characterized for mating type genes, pheromone precursors and receptors, for genes related to conidiation, for light responsive genes, and for genes showing different levels of phylogenetic affiliation (level of conservation) within eukaryotes. In general, fungal mating type has not been thought to affect individual fitness during clonal development, perhaps because morphological differences have not been observed between mating types of model filamentous fungi such as *Neurospora crassa*. However, genes close to the mating type locus have shown mating type-specific expression during sexual development in *N. crassa* (Randall and Metzenberg, 1998). Mating type genes' impacts on genes other than pheromone precursors and receptors have been reported in *Fusarium*, *Sordaria* and *Podospora* species (Pöggeler et al., 2006; Keszthelyi et al., 2007; Klix et al., 2010; Bidard et al., 2011). Our results demonstrate significant differences in gene expression between *N. crassa* strains of differing mating type during asexual development. Corresponding relations between mating types and fungal asexual development in diverse fungi have been previously reported (Kolmer and Ellingboe, 1988; Funnell et al., 2001), and, moreover, mating types are well known to exhibit differences in superiority of crossing and sexual development in *Neurospora* species (Dettman et al., 2003). In this study we made use of largely isogenic strains of *N. crassa* mating types. Sequence and hybridization data show that even the centromere-proximal flanks of the *mat A* and *mat a* idiomorphs are highly similar for *N. crassa* (Randall and Metzenberg, 1995), but full verification for the isogenic status requires genome sequencing of the *mat a* strain.

4.1. Regulatory interactions with mating type complicate conidial gene expression

Expression levels of the two best-characterized *ccgs* in *N. crassa*, *ccg-1* and *ccg-2*, appear to be regulated by non-clock controlled factors under a constant light condition. Genes *ccg-1* and *ccg-2* are known as morning-specific genes, and were well measured in this experiment (Table S2,S4). The gene *ccg-2* decreased significantly in expression in *mat a* during conidiation (Table S2, S4). Decreased expression of *ccg-2* could be associated with lower conidial dispersal; in any case, the divergent expression between mating types is challenging to explain unless mating types have a proclivity toward playing different roles during life cycle in the natural ecosystem. For example, opposite mating types growing in the same environment may time their asexual and sexual reproduction differently to maximize mating success. In a daily rhythmic light environment, expression of *ccg-1* and *ccg-2* are regulated by *frq*-oscillator and some other factors (Arpaia et al., 1995; Vitalini et al., 2004). The observed divergence in expression between mating types suggests that there are likely regulators other than *frq*, *wc-1*, and *wc-2*, for expression of *ccg-1* and *ccg-2*.

Typically, genes involved in conidia reproduction (*con*) were similarly expressed between the two mating types. Expression of three *con* genes, *con-6*, *con-8*, and *con-10* was measured across asexual development for both mating types in this study (Table S4). The

gene *con-6* is expressed upon induction of conidiation, reaching high levels at the late stages of conidiation (White and Yanofsky, 1993) and in mature conidia, but is not detectably expressed in mycelium. The down-regulated expression pattern for *con-6* found in this study was the same pattern as described by Greenwald et al. (2010), but differed from other studies that did not maintain a constant light environment (White and Yanofsky, 1993). The genes *con-8* (up-regulated) and *con-10* (no significant change) were also expressed in our study in a similar pattern to that described by Greenwald et al. (2010). For the gene *con-8*, there is no clarity about its function in conidiation and no evidence its expression is regulated by light or clock-controlled elements. Previous studies in different light environments also demonstrate that *con-8* is expressed in older cultures at a high level (Roberts and Yanofsky, 1989; Sachs and Yanofsky 1991).

4.2. Mating type genes showed no mating-type specific regulation on expression of pheromone precursors and receptors

Transcription of pheromone genes could be more complex than just mating-type specific. Although mating-type specific expression of pheromone precursors was well established with mating-type knockouts (Bobrowicz et al., 2002), co-expression of *ccg-4* and *mfa-1* has been observed in wild type strains of *mat A* and *mat a* and mutant *mat a-1*^{m33} (Zhu et al., 2001; Bobrowicz et al., 2002; Kim et al., 2002). The co-expression of both *ccg-4* and *mfa-1* observed in this study implies transcription of these genes is not solely dependent on transcription of either *mat A-1* or *mat a-1* separately. Δ*ccg-4 mat A* and Δ*mfa-1 mat a* strains completely lose male fertility, a trait attributable to the production of conidia that fail to induce trichogynes from female structures in opposite mating types (Kim et al., 2002; Kim and Borkovich, 2006). Δ*ccg-4 mat a* or Δ*mfa-1 mat A* strains exhibit normal chemotropic attraction and male fertility and show no detectable phenotypic changes in their vegetative growth (Kim and Borkovich, 2006). Examination of female fertility of those mutants revealed that Δ*ccg-4*, Δ*mfa-1* and Δ*ccg-4*Δ*mfa-1* double mutants in *mat A* and *mat a* exhibit normal protoperithecial development (Kim and Borkovich, 2006). Based on observed differential transcription patterns of pheromone receptor genes, *pre-2* and *pre-1*, in and between mating types in this study and previous studies (Pöggeler and Kück, 2001; Kim et al., 2002; Kim and Borkovich, 2004), regulation of transcription of *pre-1* and *pre-2* appears to be independent from the transcription of pheromone precursors. However, mRNA level of *pre-2* was more consistent during asexual development than *pre-1* between mating types. Unlike previous studies measuring pheromone precursors only in conidia and pre-conidiation mycelium in liquid cultures, we examined transcription of pheromone genes from the whole clone composed of a mixture of tissues undergoing vegetative growth and conidiation. Comparatively low transcription of *ccg-4* in *mat a* (about 27% of that in *mat A* at the highest expression) and high expression of *mfa-1* in *mat A* (about 72% of *mfa-1* in *mat a* at the highest expression) during asexual development deserve further investigation to better characterize basal level of expression and the extent of stochastic variation in transcription of these genes. Identification of the location of the transcripts of these genes in surface hyphae and other mycelial tissues is thus called for. Interestingly, the transcription of *mfa-1* in the *mat a* strain and its receptor *pre-1* in *mat A* strain showed the same pattern across asexual development, with peak transcription at the 60 h stage, while transcription of *ccg-4* and *pre-2* also had matching patterns, but peaked at 96 h in *mat A* and *mat a* strains. This difference in peak transcription implies that there may be different timings of mating proclivity between mating types. If such differences operate in nature, it would challenge the paradigm of the independence of genetic mating type (*mat A*, *mat a*) and phenotypic gender (conidia or protoperithecia as in *Neurospora crassa*) in fungi. However, pre-pro-pheromones encoded by *ccg-4* or *mfa-1* in ascomycetes are translated and further processed to become mature pheromones excreted by appropriate cells (Pöggeler, 2011), and post-transcriptional repression of the expression of pheromone genes by opposite mating type was demonstrated

in *P. anserina* (Coppin et al., 2005). Processing of pre-pro-pheromones in *N. crassa* is not yet well annotated, and transcription of many genes similar to those involved in *S. cerevisiae* pheromone processing, including endopeptidase (NCU03219), carboxypeptidase (NCU04316), and farnesyltransferase (NCU05999), was not detected in this study. However, transcription of genes similar to *S. cerevisiae* pheromone processing genes *ram2* (NCU03632), *ste13* (NCU02515), *ste14* (NCU00034), *ste24* (NCU03637), and *AXL1* (NCU00481), was well measured, and transcription of NCU03637, NCU00034, and NCU00481 all showed significant increase during the asexual development in both mating-types (Table S1). Thus, further investigation of pheromone molecule concentration is required to test if transcription levels of pheromone precursors measured in this study directly reflect the protein level of mature pheromones in the system.

As our experiment was performed with samples exposed to constant light and temperature, the impact of the circadian clock on pheromone precursor genes is presumably muted. The overall transcription of mating locus and corresponding pheromone precursor genes and receptor genes was, however, clearly correlated with development stages. Intriguingly, these mating-type specific genes exhibit dramatic changes in transcription during “asexual” development. Accordingly, late asexual development may best be regarded as a time of pre-sexual development, where genetic preparation for mating and sexual reproduction starts. However, development of protoperithecia in *N. crassa* requires different growth conditions (Adomas et al., 2010), and we did not include any samples of protoperithecia in this study.

4.3. Functional category classification implies a need for further evaluation of the effects of mating type in studies of asexual development

Enrichments of genes in functional categories observed in this experiment imply distinct genetic regulatory mechanisms in *mat A* and *mat a* strains, mechanisms which up-regulate certain groups of genes during colony expansion in *mat A* genotypes, and mechanisms that down-regulate other groups of genes in *mat a* genotypes. Most genetic studies with *Neurospora crassa* have been carried out using the *mat A* mating type strains with little concern for the effect of this choice of genetic locus for sexual identity. However, our study shows that “asexual” development of *N. crassa* does have cryptic “sexual” components. These subtle differences associated with mating type will have implications for designing experiments for studying the genetic basis of asexual phenomena, such as conidiospore formation and protoperithecia development.

4.4. Expression of early and late light responsive genes during clonal development

Early and late light responsive genes are expressed differently during clonal development in both mating types. Light is suggested to play a critical role, via circadian systems in the mediation of sexual and asexual development in *N. crassa*, and the genetic basis of light responses in fungi has been studied for decades (Dunlap and Loros, 2004; Purschwitz et al., 2006, 2008; Chen et al., 2009). A constant light condition is generally used in the study of reproduction and associated morphological development in *N. crassa* and some other fungi (Hallen et al., 2007; Kasuga and Glass, 2008), yet the systemic effect of a constant light environment on *N. crassa* has not been examined. Underlying the complicated nature of biological reaction toward light, about 340 early light responsive genes (ELRGs) and late light responsive genes (LLRGs), over 92% of the total identified light-responsive genes, exhibit demonstrated changes in response to light (Chen et al., 2009). In experimental exposures to 4 hours of constant light following a long period of constant darkness, the ELRGs peaked between 15 and 45 min after the onset of light, and LLRGs peaked later: between 45 and 90 min (Chen et al., 2009). Although expression of *wc-1*, *wc-2*, and related *frq* were not measurably detected in this experiment, differences in expression patterns across asexual development between ELRGs and LLRGs were clear (Fig. 3, Table S5,S6).

We found that some LLRGs appear to be critical for late asexual development in *Neurospora crassa*. Expression of LLRGs is suggested to be regulated by light, probably via ELRGs (Chen et al., 2009). Via these mechanisms, the fungus is capable of sensing the light environment in a daily manner and is prepared to respond developmentally when appropriate levels of light and/or an appropriate stage of asexual development are reached. In *Fusarium verticillioides*, a fungal pathogen closely related to *N. crassa*, transcript levels of genes involved in photo-inducible carotenoid biosynthesis and of a green-light sensor similar gene *carO* were significantly reduced in a •FvMAT1-2-1 (*mat a*) knockout mutant (Keszthelyi et al., 2007; Adám et al., 2011). Our data showed a significant down-regulation for a carotenoid biosynthesis gene *al-1* (NCU00552) in *mat A*, while a consistent expression level of this gene in *mat a* during the asexual development in *N. crassa*.

To summarize, mating loci were expressed at increasing levels during asexual development, and expression of pheromone precursors *ccg-4* and *mfa-1* and receptors *pre-1* and *pre-2* were detected in both mating types in all development stages. Moreover, expression of mating-type-specific pheromone-related genes occurred at different levels in the two mating types. This mating-type specific pheromone-related gene expression may relate to divergent roles of mating types in pre-sexual development and crossing. These divergent roles are also supported by the observation of significant differences in overall gene expression between the mating types across asexual development, especially at the late development stage prior to sexual differentiation. The *mat A* strain tended to exhibit heightened expression for many of the differentially expressed genes, and typically exhibited more dynamic regulation of gene activity. As we did not use completely isogenic strains of the two different mating-types, some of the differences observed may be attributed to other genetic variations between the two strains. Our results also call for further investigation of the impact of light and roles of light response genes in asexual development in this fungus, given significant up-regulation of expression was observed for many late light responsive genes at late asexual development stages in both mating types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

> Genome-wide, genes in mat A more frequently exhibited a higher expression level than genes in *mat a*. > Genome-wide, mat A demonstrated greater transcriptional regulatory dynamics. > Mating loci were increasingly expressed across asexual development for both mating types. > Pheromone and receptor genes were expressed within the whole clones for both mating types. > Early and late light responsive genes showed similar expression timing over asexual development.

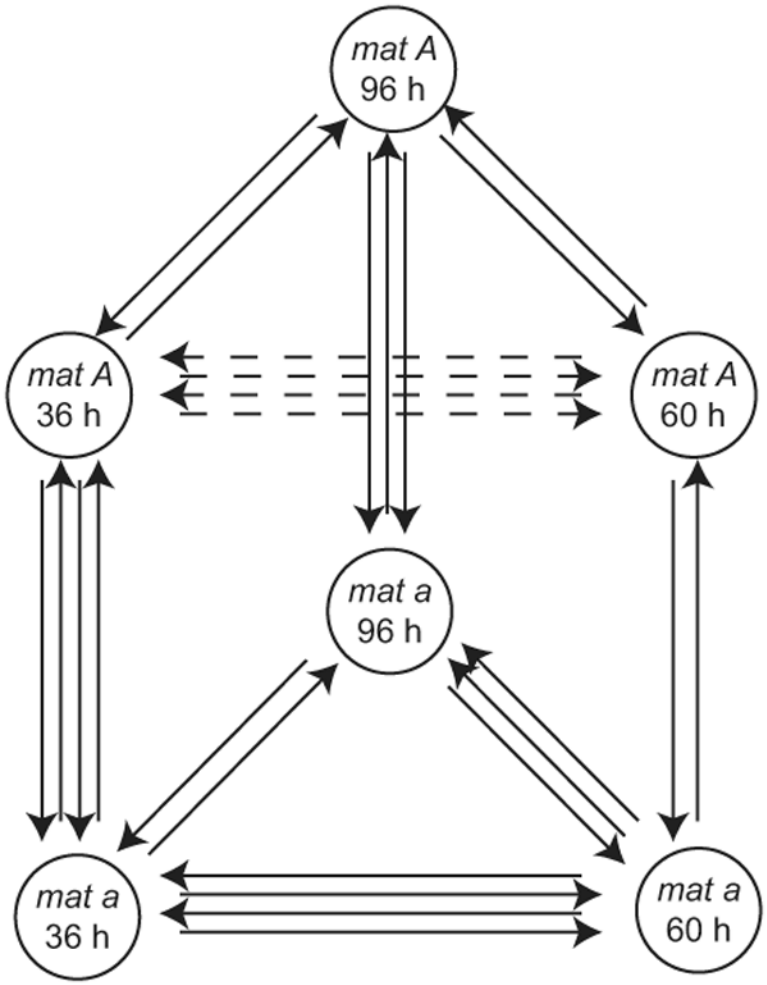

Figure 1.

Experimental design for microarray comparisons of sex-specific gene expression during asexual development of *Neurospora crassa*. Each arrow represents an independent reverse transcription and microarray hybridization from numerous pooled biological replicates. Each arrowhead corresponds to the sample labeled with cyanine 5-dUTP, and each blunt end corresponds to the sample labeled with cyanine 3-dUTP. All samples were compared directly to neighboring time points, and the circuit design was closed by direct comparisons of 36 h to 96 h samples. Additionally, all time points featured direct comparisons between samples from strains of opposite mating type.

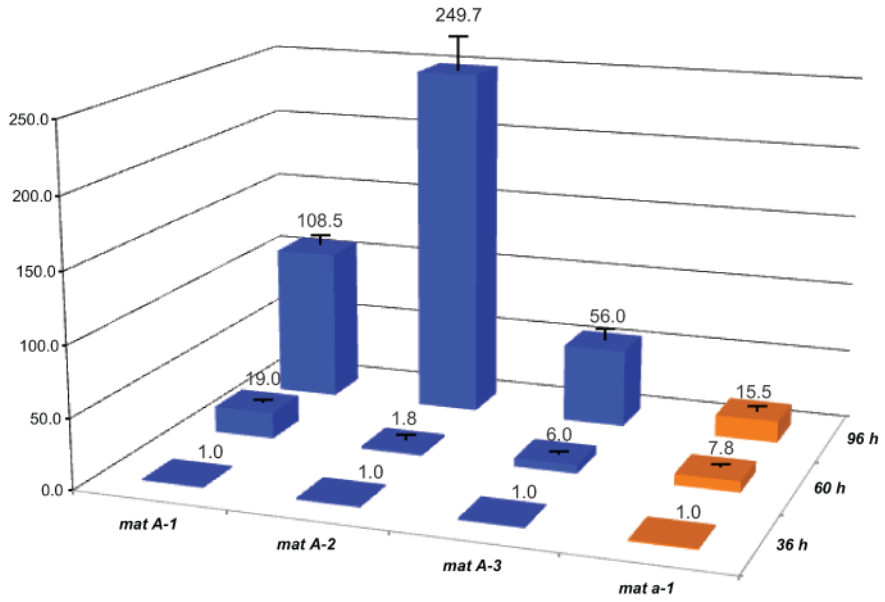

Figure 2.
Real-time PCR estimates of the expression of *mat* genes in *mating type A* and *a* strains of *Neurospora crassa* at three time points during asexual development.

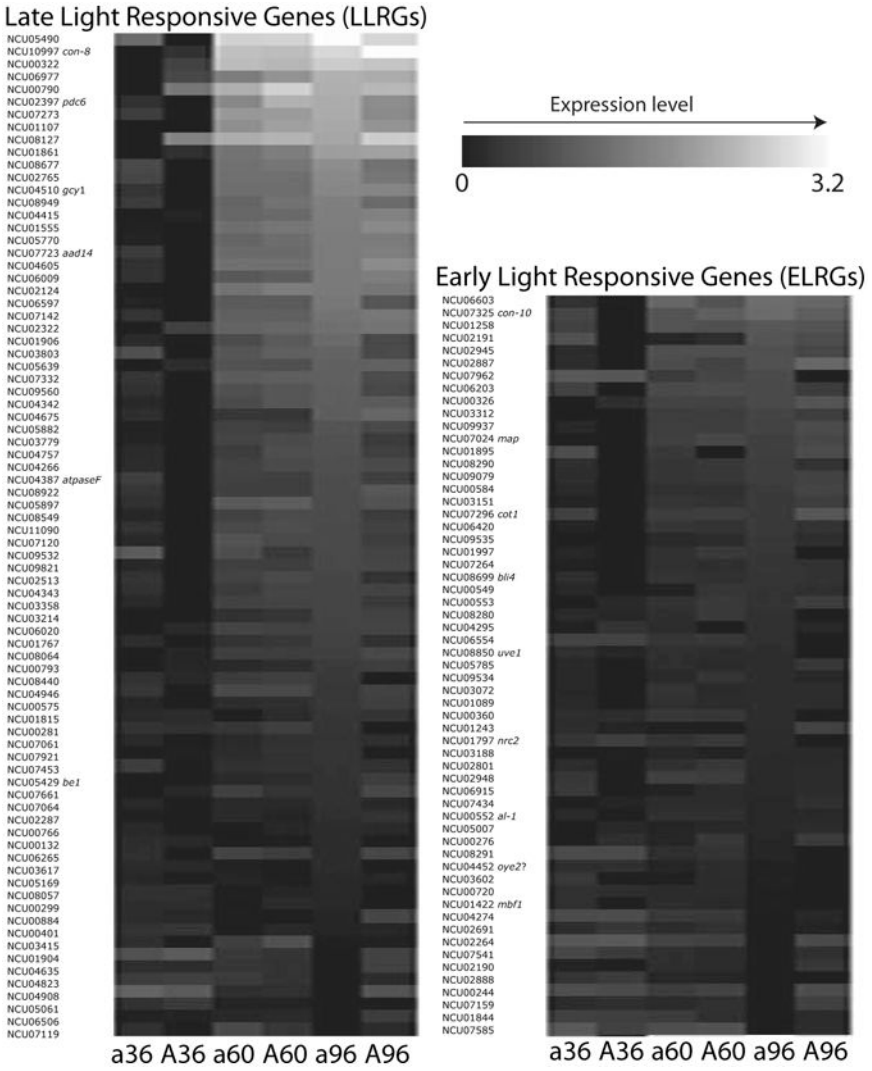

Figure 3.

Heat maps depicting comparative expression level ($\log_2$ ratio) of the early light responsive genes (ELRGs, Chen et al., 2009) and the late light responsive genes (LLRGs, Chen et al., 2009) in strains of *mating type A* and *a* of *Neurospora* across three time points of asexual development. Lighter color corresponds to a higher level of expression.

Table I

Numbers of genes well measured, and statistically significant, across the whole data set as well as subsets of nodes in the hybridization design.

Feature	Whole set		36-60 h		60-96 h		A only		a only	
Well-measured genes	4491		4296		3403		3848		3553	
Gene detected only in the subset	NA*		106		58		86(32)#		11(6)#	
Mating type	A	a	A	a	A	a	A	a		
Genes significantly differentially expressed (p•0.05)	2403	2012	1544	1411	1307	147	1601	1018		
Up-regulated genes for 36<60<96 (p•0.05)	79	64	NA	NA	NA	NA	50	24		
Down-regulated genes for 36>60>96 (p•0.05)	66	102	NA	NA	NA	NA	31	39		
Up-regulated genes for 36<60 (p•0.05)	1190	1000	972	893	NA	NA	688	510		
Down-regulated genes for 36>60 (p•0.05)	587	574	573	520	NA	NA	376	315		
Up-regulated genes for 60<96 (p•0.05)	289	195	NA	NA	441	11	224	79		
Down-regulated genes for 60>96 (p•0.05)	917	136	NA	NA	866	136	656	218		
GEL_{50}	1.35	1.37	1.36	1.36	1.62	2.09	1.51	1.64		

*Not applicable.

#Number of additional genes not detected as expressed at all time points; 36-60 h and 60-96 h results.

Table II

Relative expression levels of pheromone precursors/receptors estimated with quantitative RT-PCR.

Gene	NCU	*mat a* 36 h	*mat a* 60 h	*mat a* 96 h	*mat A* 36 h	*mat A* 60 h	*mat A* 96 h
ccg-4	NCU02500	2.71±1.20	1.00	2760±1397	45.15±19.27	131.69±43.28	10078±3507
mfa-1	NCU01257	1.00	56.57±5.53	15.15±6.33	3.07±0.90	4.11±0.25	41.32±5.23
pre-1	NCU00138	1.00	2.30±0.36	14.96±7.06	2.01±0.72	8.09±1.45	5.57±1.02
pre-2	NCU05758	1.00	1.05±0.08	2.32±1.03	1.21±0.40	1.38±0.49	1.52±0.47

Table III

Table III-A. Functional categories that were enriched for genes exhibiting increasing expression, for each mating type.

Total number of genes in A = 78 out of 79, and in a =61 out of 64 annotated in FunCat.	**No. of genes in A**		**No. of genes in a**		**P-value**	
Functional Category	Observed	Expected	Observed	Expected	*mat A*	*mat a*
01.25.01 extracellular polysaccharide degradation	3	0.1404	0	NA	0.0004	NA
01.04.04 regulation of phosphate metabolism	3	0.1482	0	NA	0.0005	NA
01.05.03 polysaccharide metabolism	6	1.3026	3	1.83	0.0019	0.0827
01.25 extracellular metabolism	3	0.3042	0	NA	0.0036	NA
32.10.07 degradation / modification of foreign (exogenous) polysaccharides	2	0.0936	0	NA	0.0044	NA
01.05 C-compound and carbohydrate metabolism	13	5.8812	7	4.27	0.0052	0.1739
30.01.05.01.06 serine/threonine kinase	2	0.1326	1	0.61	0.0084	0.1037
20.03 transport facilities	7	2.3478	4	2.44	0.0089	0.1110
32.10 degradation / modification of foreign (exogenous) compounds	2	0.1482	0	NA	0.0103	NA
01.06.10 regulation of lipid, fatty acid and isoprenoid metabolism	2	0.1638	0	NA	0.0124	NA
34.01.01.01 homeostasis of metal ions (Na, K, Ca etc.)	4	0.9048	0	NA	0.0128	NA
30.01.05.01.02 JNK cascade	1	0.0078	0	NA	0.0154	NA
32.05.01.03 chemical agent resistance	2	0.195	0	NA	0.0171	NA
10.03.02 meiosis	3	0.5772	0	NA	0.0203	NA
34.11.03 chemoperception and response	5	1.6146	3	1.83	0.0229	0.1330
01.04 phosphate metabolism	6	2.223	4	2.44	0.0236	0.0957
20.01.01.07 anion transport	2	0.234	0	NA	0.0238	NA
20.01.01 ion transport	4	1.1544	1	0.61	0.0283	0.5985
16.17.03 potassium binding	1	0.0234	0	NA	0.0306	NA
20.01.01.07.09 chloride transport	1	0.0234	0	NA	0.0306	NA
30.05.01.18 transmembrane receptor protein serine/threonine kinase signaling pathways	1	0.0234	0	NA	0.0306	NA
02.25 oxidation of fatty acids	2	0.2886	0	NA	0.0348	NA
10.03.01.01.11 Mphase	2	0.3354	0	NA	0.0455	NA
20.03.01 channel / pore class transport	2	0.2028	3	1.83	0.0184	0.0006
32.07.07 oxygen and radical detoxification	1	0.234	3	1.83	0.2146	0.0008
01.20.17.09 metabolism of alkaloids	2	0.1092	2	1.22	0.0058	0.0036

Table III-B. Functional categories that were enriched for genes exhibiting decreasing expression, for each mating type.

Total number of genes in A = 61 out of 66, and in a = 95 out of 102 annotated in FunCat.	No. of genes in A		No. of genes in a		P-value	
Functional Category	Observed	Expected	Observed	Expected	*mat A*	*mat a*
32.05.05 virulence, disease factors	1	0.366	4	3.8	0.311	0.003
01.01.09.01.02 degradation of glycine	0	NA	2	1.9	NA	0.004
01.01.09 metabolism of the cysteine - aromatic group	1	0.6832	5	4.75	0.499	0.004
20.01.07 amino acid/amino acid derivatives transport	1	0.2135	3	2.85	0.197	0.005
01.01.06.04 metabolism of threonine	1	0.0671	2	1.9	0.070	0.005
20.03.02.02.02 sodium driven symporter	0	NA	1	0.95	NA	0.009
01.01.06.06.01 biosynthesis of lysine	1	0.0915	2	1.9	0.093	0.010
01.01.09.01 metabolism of glycine	0	NA	2	1.9	NA	0.011
01.06.02.02 glycolipid metabolism	0	NA	2	1.9	NA	0.011
01.01.09.02 metabolism of serine	1	0.1098	2	1.9	0.109	0.014
01.01.06.06 metabolism of lysine	1	0.1586	2	1.9	0.152	0.027
01.01 amino acid metabolism	1	1.9276	7	6.65	0.861	0.031
14.13.01 cytoplasmic and nuclear protein degradation	1	0.7686	4	3.8	0.540	0.032
01.01.06.05 metabolism of methionine	1	0.183	2	1.9	0.172	0.034
42.10.07 nucleolus	0	NA	1	0.95	NA	0.037
01.01.06.04.01 biosynthesis of threonine	1	0.0244	1	0.95	0.030	0.046
34.01.03.03 homeostasis of phosphate	0	NA	1	0.95	NA	0.046
20.09.04 mitochondrial transport	4	0.549	2	0.855	0.002	0.212
20.03 transport facilities	6	1.8361	4	2.8595	0.010	0.321
14.07.02.02 N-directed glycosylation, deglycosylation	2	0.1525	0	NA	0.011	NA
01.05.03 polysaccharide metabolism	4	1.0187	4	1.5865	0.019	0.076
02.07.01 pentose-phosphate pathway oxidative branch	1	0.0244	0	NA	0.030	NA
32.07.07.05 peroxidase reaction	1	0.0244	0	NA	0.030	NA
34.07.01 cell-cell adhesion	1	0.0305	0	NA	0.036	NA
20.09 transport routes	8	3.8491	7	5.9945	0.037	0.394
01.05 C-compound and carbohydrate metabolism	9	4.5994	10	7.163	0.038	0.178
14.07.02 modification with sugar residues (e.g. glycosylation, deglycosylation)	2	0.3355	0	NA	0.045	NA
20.01 transported compounds (substrates)	8	4.0077	8	6.2415	0.045	0.286
20.03.02.03.01 proton driven antiporter	1	0.0427	0	NA	0.047	NA

Table IV

Expression patterns of genes with different levels of phylogenetic conservation.

Phylogenetic classes of *N. crassa*genome (9127 genes)*	No difference between Mat-A and -a	Mat-A no change		Mat-a no change		Other differences	SUM**	
		Mat-a up	Mat-a down	Mat-A up	Mat-A down			
NC orphans (24%)	301	14	21	48	43	60	186 (11%)	487 (12%)
Pezizo-specific (35%)	701	39	79	127	84	151	480(27%)	1181 (28%)
Ascomycota-core (2%)	38	1	4	5	2	8	20 (1%)	58 (1%)
Dikarya-core (11%)	301	21	32	50	52	63	218 (11%)	519 (12%)
Euk/Prok-core (26%)	1000	64	164	194	123	257	802 (47%)	1802 (43%)
others (2%)	52	4	7	12	9	15	47 (3%)	99 (2%)
		143 (8%)	307 (18%)	388 (24%)	313 (18%)	554 (32%)	1705	
	2339 (59%)	143 (3%)	307 (8%)	388 (9%)	313 (8%)	554 (13%)	4146 analyzed	

*Follows Kasuga et al. (2009).

**Percentage of genes detected for each category was calculated based on total genes analyzed (4146 genes) and genes (highlighted) showing different expression patterns between mating types (1705 genes).