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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 in strains 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.

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 (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 *Neurospora 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.

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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 MATα-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., 2002; 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 *P. anserina* and *S. macrospora*, mating type specific expression was observed for genes with diverse function, including metabolism, 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. While the 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 14 μmol/m^2 S at the media surface, measured at wavelengths between 400 nm and 700 nm. Mycelia were harvested from 90 mm Petri dishes at 36 h, 60 h, and 96 h, corresponding to vegetative growth (colony-size 30–50 mm), start to middle of conidia production (50–70 mm), 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