Complex Mechanisms Regulate Developmental Expression of the *matA* (HMG) Mating Type Gene in Homothallic *Aspergillus nidulans*

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ABSTRACT Sexual reproduction is a fundamental developmental process that allows for genetic diversity through the control of zygote formation, recombination, and gametogenesis. The correct regulation of these events is paramount. Sexual reproduction in filamentous fungi, including mating strategy (self-fertilization/homothallism or outcrossing/heterothallism), is determined by the expression of mating type genes at *mat* loci. *Aspergillus nidulans matA* encodes a critical regulator that is a fungal ortholog of the hSRY/SOX9 HMG box proteins. In contrast to well-studied outcrossing systems, the molecular basis of homothallism and role of mating type genes during a self-fertile sexual cycle remain largely unknown. In this study the genetic model organism, *A. nidulans*, has been used to investigate the regulation and molecular functions of the *matA* mating type gene in a homothallic system. Our data demonstrate that complex regulatory mechanisms underlie functional *matA* expression during self-fertilization and sexual reproduction in *A. nidulans*. *matA* expression is suppressed in vegetative hyphae and is progressively derepressed during the sexual cycle. Elevated levels of *matA* transcript are required for differentiation of fruiting bodies, karyogamy, meiosis, and efficient formation of meiotic progeny. *matA* expression is driven from both initiator (Inr) and novel promoter elements that are tightly developmentally regulated by position-dependent and position-independent mechanisms. Deletion of an upstream silencing element, *matA* SE, results in derepressed expression from wild-type (wt) promoter elements and activation of an additional promoter. These studies provide novel insights into the molecular basis of homothallism in fungi and genetic regulation of sexual reproduction in eukaryotes.

SEXUAL reproduction is a central part of the life cycle in most eukaryotic organisms and has a profound impact on the evolution and biology of species. Successful exchange of genetic material requires establishment of sexual identity (male and female function). Sexual identity in animals is governed by genes located on sex chromosomes, whereas in fungi mating type genes residing at *mat* loci (sexual-identity loci) control cell-type identity and sexual development. The correct regulation and spatiotemporal expression of sex determining genes is crucial during reproduction. Mechanisms underlying gene regulation in eukaryotic sexual development are not well understood (Harley *et al.* 2003; Fraser and Heitman 2005; Kashimada and Koopman 2010). Mating type genes have been described in numerous heterothallic (cross-fertile) and homothallic (self-fertile) filamentous fungi, where they function as master regulators of sexual reproduction (Kronstad and Staben 1997; Debuchy *et al.* 2010). The *mat* loci typically encode transcription factors, either Mat-HMG (high-mobility group box) or Mat-α (alpha box), proteins that coordinate expression of sex-specific genes. Several lines of study indicate that mating type genes in fungi share structural and functional features with the more complex sex chromosomes of algae, plants, and animals (Kronstad and Staben 1997; Marais and Galtier 2003; Fraser and Heitman 2004, 2005; Debuchy *et al.* 2010). It has also been suggested that Mat-HMG in fungi represents a structural ortholog of the human SRY regulator (sex-determining region Y) (Fraser and Heitman 2004, 2005; Fraser *et al.* 2005, 2007; Heitman 2006).

The genetic organization of mating type genes at *mat* loci determines sexual identity and mating strategy in filamentous fungi (Nelson 1996; Coppin *et al.* 1997; Debuchy *et al.*

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2010). In heterothallic fungi, the *mat* locus typically encodes one of two nonallelic sequences (idiomorphs) that occupy the same genetic position on homologous chromosomes in two cross-compatible mating strains, termed *mat-HMG* (+) and *mat*-α (−). Idiomorphic structure of *mat* loci governs pheromone signaling and cell-specific gene expression in sexually compatible mating strains. Fertilization occurs exclusively between *mat(+)* and *mat(−)* strains and self-fertilization is prohibited (Turgeon 1998). The role of the mating type genes has been well characterized in outcrossing heterothallic systems, where Mat proteins control not only recognition mechanisms leading to cross-fertilization but also later stages of sexual development, including nuclear segregation and coordinated fruiting body differentiation (Metzenberg and Glass 1990; Debuchy and Turgeon 2006). In contrast to heterothallic species, most homothallic fungi have closely linked *mat-HMG* and *mat-α* genes present in the haploid genome. This genetic organization confers self-compatible (self-fertile) mating ability (Turgeon 1998; Yun *et al.* 2000; Debuchy and Turgeon 2006). An exception is self-fertile *Neurospora africana*, which has only *mat A-1* (α box) present in the genome (Glass and Smith 1994). Homothallic species such as *Aspergillus nidulans* are also cross-fertile. Genetic analysis of outcrosses between self-fertile *A. nidulans* strains led Pontecorvo *et al.* (1953) to propose the term "relative heterothallism" to explain the ability of a homothallic species to behave as if it was heterothallic. However, a role for *mat* loci in discrimination and recognition between mating partners is poorly understood and remains elusive (Pontecorvo *et al.* 1953; Hoffmann *et al.* 2001). Several studies suggest that sexual identity in homothallic systems might be regulated by differential expression of mating type genes or the molecular "transformation" of the homothallic *mat* loci into a heterothallic state by selective epigenetic silencing of one of the two mating type genes (Metzenberg and Glass 1990; Raju and Perkins 2000; Fraser and Heitman 2004; Scazzocchio 2006).

It has been previously demonstrated that the HMG-box mating type transcription factor is necessary for fruiting body development in both heterothallic and homothallic fungi. Mat-HMG is required for correct expression of different sets of target genes directly involved in male and female fertility, fruiting body morphogenesis, fruiting body abundance, and ascospore formation (Coppin *et al.* 1997; Kronstad and Staben 1997; Debuchy 1999; Debuchy *et al.* 2010) Surprisingly, very little is known about the mechanisms that govern regulation and transcriptional expression of mating type genes in fungi.

A. nidulans is a homothallic filamentous fungus with unlinked *mat* loci, *matA* (HMG box) and *matB* (α box). In this study we analyzed the regulation and molecular function of the *matA* mating type gene during the self-fertile sexual cycle. Sexual development in *A. nidulans* involves coordinated differentiation of three multicellular tissue types: Hülle cells, ascogenous hyphae, and the cleistothecium wall. Hülle cells appear at the onset of the sexual cycle and function as nurse cells to protect unfertilized female organs (protocleistothecia) and provide nutrients for the developing fruiting body (cleistothecium) and ascospores. Sexual conjugation (mating) is believed to occur within clusters of Hülle cells, where cellular equivalents of female and male reproductive structures fuse and combine nuclei within a common cytoplasm during the process of fertilization. After fertilization, parental haploid nuclei divide synchronously in the female organ and are ultimately segregated into dikaryotic cells of proliferating ascogenous hyphae. Dikaryotic cells differentiate by a coordinated series of cell and nuclear divisions to form ascus mother cells, where nuclear fusion (karyogamy) is followed by meiosis. The differentiation of sexual spores (ascospores) occurs within these enclosed structures (asci). Concurrent with development of reproductive hyphae, vegetative aerial hyphae grow in circular fashion surrounding the fertile ascogenous tissue and eventually form the hard cleistothecial wall (Benjamin 1955; Champe *et al.* 1994; Turgeon 1998; Bruggeman *et al.* 2003; Pyrzak *et al.* 2008).

On the basis of gene deletion/complementation studies and the analysis of the transcriptional regulation, we have determined that unique, position-dependent and position-independent mechanisms regulate *matA* developmental expression and that the level of *matA* expression is a critical modulator of self-fertility. *matA* expression is suppressed in vegetative hyphae and is progressively derepressed in the developing sexual reproductive tissue. Vegetative suppression appears to be regulated by an upstream silencer element (SE). Deletion of the silencer, *matA* SE, resulted in the dramatic derepression/upregulation of the *matA* transcript in vegetative tissue and approximately threefold depression in developmental tissue. Additionally, we identified a conserved element (29-bp sequence) with striking structural similarity to the silencer of the human *SRY* gene. However this element did not have an effect on *matA* regulation. We did not find that vegetative suppression of *matA* is required for self-fertility. By contrast, an increase in *matA* transcript during sexual development is critical for efficient fruiting body differentiation and formation of meiotic progeny. Developmental upregulation appears to be driven by novel promoter elements and potential Inr (initiator) sequences located at the sites of transcriptional imitation. These promoter elements do not require other typical upstream promoter elements such as TATA or CAAT boxes, or fungal pyrimidine tracts. Highest *matA* expression was detected in reproductive tissue and coincided with the stage of rapid differentiation of asci and ascospores. Failure to upregulate *matA* transcript resulted in aborted formation of ascospores. Therefore, increasing *matA* transcript abundance is required for progression through specific developmental stages. We found that *matA* expression is tightly regulated by both position-dependent and position-independent mechanisms. An unusual feature of the position-dependent regulation is that duplication of the 5′ genomic region flanking *matA*, which includes the silencer element, correlates with

Table 1 *A. nidulans* strains used in this study

Strain	Genotype	Source
FGSC A26	biA1	Fungal Genetics Stock Center
GR5	pyrG89; wA3; pyroA4	May, G. S. (M. D. Anderson)
UI427	alcA(p):medA, biA1, pyrG89:matA:pyrG, pabaA1; wA1; argB2; matAΔ::argB,	This study
UI461	yA2, pabaA1, pyroA4; argB2; matA(0)::AfargB; nkuAΔ^{ku70}::bar	This study
UI462	yA2, pyrG89, pabaA1; nkuAΔ^{ku70}::bar; argB2; matA(0)::AfargB	This study
UI465	yA2, pyrG89, pabaA1; argB2; matA(0)::AfargB	This study
UI465 3.2	yA2, pyrG89:pWP3^{-898};pyrG, pabaA1; argB2; matA(0)::AfargB	This study
UI465 3.5	yA2, pyrG89:pWP3^{-248}:pyrG, pabaA1; argB2; matA(0)::AfargB	This study
UI465 3.6	yA2, pyrG89:pWP3^{-512}:pyrG, pabaA1; argB2; matA(0)::AfargB	This study
UI467	yA2, pyrG89:pWP3^{-170}:pyrG, pabaA1; argB2; matA(0)::AfargB,	This study
UI468	yA2, pyrG89:pWP3$^{\Delta 170}$:pyrG, pabaA1; argB2; matA(0)::AfargB	This study
UI472	yA2, pyG89:pWP3^{-1001}:pyrG, pabaA1; argB2; matA(0)::AfargB	This study
FGSC A1153	yA2, pabaA1, pyroA4; argB2; nkuAΔ^{ku70}::bar	Hynes, M, Fungal Genetics Stock Center
FGSC A1155	pyrG89, pyroA4; nkuAΔ^{ku70}::bar	Hynes, M. Fungal Genetics Stock Center

position-dependent suppression of *matA* expression during development and negatively impacts ascospore formation.

Our data demonstrate that self-compatible *A. nidulans* has complex regulatory mechanisms controlling functional expression of the HMG-box mating type gene. Further investigation of mating type gene regulation in *A. nidulans* may reveal mechanisms controlling recognition between sexually compatible cells and nuclei in the homothallic mating system. Understanding fungal mating type gene regulation may also provide novel insights into those pathways conserved throughout eukaryotic sexual reproduction as well as mechanisms that generate diversity in reproductive strategies.

Materials and Methods

Strains, growth conditions, and genetic manipulations of A. nidulans

A. nidulans strains used in this study are listed in Table 1. Supplemented minimal and complete media were prepared as previously described (Pontecorvo *et al.* 1953; Kafer 1977; Vallim *et al.* 2000). Standard genetic techniques were used as described previously (Pontecorvo *et al.* 1953). Nucleic acid DNA and RNA isolation, Southern blot, Northern blot analysis, and standard molecular techniques were performed as previously described (Miller *et al.* 1985, 1987; Wu and Miller 1997). DNA-mediated transformation of *A. nidulans* was performed according to previous protocols (Yelton *et al.* 1983; Miller *et al.* 1985). Induction of sexual development and fruiting body analysis in *A. nidulans* were performed as described by Pyrzak *et al.* (2008) and Vallim *et al.* (2000).

Light microscopy

Photomicrographs were taken with a Nikon Cool Pix 5400 camera and Zeiss SV8 stereomicroscope. Fruiting bodies were cleaned, suspended in sterile water on glass slides, and crushed under a coverslip. Fruiting body tissue was examined by differential interference contrast (DIC) optics using a Zeiss Axioplan microscope. Photographs were taken with either a Nikon Cool Pix 5400 or a Photometrics CoolSnap ES camera with Metavue software (Universal Imaging).

Comparative RT–qPCR transcript analysis

The relative RT–qPCR method was used to assess the developmental expression of *matA* transcript. Total RNA was extracted from undifferentiated hyphae and reproductive tissue at different time points. Undifferentiated hyphae tissue was obtained from a submerged culture of *A. nidulans* grown for 16 hr at 37°. Reproductive tissues were grown on a solid medium as confluent cultures and collected 2, 4, and 6 days postinduction of sexual development. Total RNA was extracted as previously described (Vallim *et al.* 2000) and treated with DNase-I to remove traces of genomic DNA contamination. To quantify expression, mRNA was reverse transcribed with oligo dT primers using the SuperScript First-Strand Synthesis system for RT–PCR (Invitrogen). Transcript levels were quantitated using the threshold cycle ($\Delta\Delta CT$) method and SYBR green sequence detection was performed using a StepOne real-time PCR system (Applied Biosystems). Relative quantitation, by the ($\Delta\Delta CT$) method, is expressed as a difference in target gene expression with respect to an internal control (actin) in different samples. The actin primers used do not span introns. Nonreverse transcribed controls for every RNA preparation were included to confirm that contaminating genomic DNA did not bias cDNA quantification. Mean CT values and standard deviations were used in $\Delta\Delta CT$ calculations. Primers used in RT–qPCR are listed in Table 2. Each cDNA sample was assayed in triplicate and RNAs were obtained from three separate biological samples. We performed control experiments comparing *argB*, *benA*, and *actA* transcripts using RNAs extending over the developmental time course. These have all been previously used as internal controls for Northern blot analysis in *A. nidulans*. Our results found that *argB* and *actA* appeared to be most constant during development. Actin has been consistently used as internal control in other organisms for RT–qPCR experiments including the filamentous fungi *A. fumigatus* and *Magnaporthe oryzae*. General methods have been described previously (Pyrzak *et al.* 2008).

Table 2 Primers used in this study

Primer	Sequence
An/AfactinF	GTCAAGATCATTGCTCCTCCTAGA
An/AfactinR	TACTCCTGCTTGGAGATCCACAT
AnMatAF11	TGGGAGTGTATCAGCTTCATG
AnmatAR11	TGCCGTATGCTACCTGAG
AnmatAF33	CCGCACGCATCACCGAGCTCC
AnmatAR29	GGTGTGCGCAGAACACGCAGA
AnmatAF31	CATCAGCGAGCGTCCATGTCGGAGGCTTTA
AnmatAF32ΔmatA	CATTGACGACCTAGTTTGGTG
AnmatAR35AfargBext	GTATAATTGGGCTGTTGGCGGTGCTACGTATCTAGGACGAACGAGTCAAGT
AnmatAF36AfargBext	ACTAAGAGCTCGTTAAGTTGTCCCCTTCCAGGGACGACTGTATGCACTCTC
AnmatAF40	AGTCTTGAGCCATGAGTTGGTAGC
AnmatAR5	CATCATTCGTAGACCCCAGATGACGCAAAG
AnmatAR6	TATCCACGACATTTGTTGGCAAAAGGACTT
GeneRacer 5′ primer	CGACTGGAGCACGAGGACACTGA
GeneRacer 5′ nested primer	GGACACTGACATGGACTGAAGGAGTA
AfargBF1	TACGTAGCACCGCCAACAGCC
AfargBR1	TGGAAGGGGACAACTTAACGA
AnmatAF38	CACAGTCTTCTGAGACACCATCTC
AnmatAR38	GCTTATTGATTCCGAAAGTGGAAG
pWp3matAF1	CTGTATCGATTGCTATGGAAATCACCAACAC
pWp3matAR1	CAGCCATTTTGGCACTTC
AnmatAF30	GCACCTCGTCTCACTCCAGATTACT
AnmatAR27	CAGAAGGCTTCCGAGGAGTGTAC
AnmatSE2F	GTATCCGTCGAGCCTACTCCT

Mapping of matA mRNA cap sites

RNA start sites were mapped using the Invitrogen Gene Racer kit, L150202. The capped transcripts were identified according to the manufacturer's instructions. Nested *pcr* fragments were produced by first using the Gene Racer 5′ primer and AnmatAR6 and then reamplifying the product with the Gene Racer 5′ nested primer and AnmatAR5. The *pcr* products were separated on a gel and isolated bands were excised. Gel fragments were purified and cloned into a Zero Blunt topo vector. The resulting construct was cloned into TOP10 chemically competent cells and plated on L-ampicillin plates. DNA from individual colonies was sequenced using an Applied Biosystems 3730 sequencer.

Gene deletion and complementation analysis

Standard gene deletion and complementation analysis were used to elucidate the molecular function and developmental regulation of the *matA* gene. An *A. nidulans matA*(0) deletion strain was created by deletion/replacement of the *matA* entire transcriptional unit (from +2 to +1851 nt) at the resident *matA* locus on chromosome (chr) III. A fusion PCR approach was used to create a deletion construct according to the protocol developed by Szewczyk *et al.* (2006). The *A. fumigatus argB* selectable marker (*AfargB*) gene was amplified from genomic DNA of *A. fumigatus* strain (Af293.139) with primers AfargBF1 and AfargBR1 (Table 2). The upstream 5′ and downstream 3′ genomic fragments flanking the *matA* transcriptional unit were amplified using primers that introduce 30-nt overlapping extensions with the selectable marker, *AfargB*. The 5′ flank was amplified with primers AnmatAF11 and AnmatAR35AfargBext (Table 2). The 3′ flank was amplified using primers AnmatAF36AfargBext and AnmatAR11 (Table 2). The PCR fragments were subsequently fused into a final *matA* deletion fragment using AnmatAF11 and AnmatAR11 primers. The final fusion product was directly transformed into Fungal Genetics Stock Center (FGSC) A1153 ($nkuA^{ku70\Delta}$) *A. nidulans* recipient strain and resulted in high frequency of homologous gene targeting. Transformants were recovered on selective media and the complete deletion of the *matA* transcription unit was confirmed by Southern blot analysis and RT–PCR in >20 individual isolates (data not shown). Plasmid pWP3 carries the coding region of *matA* flanked by 1-kb upstream and 1.8-kb downstream genomic sequences plus *pyrG* as a prototrophy marker. pWP3 was constructed by cloning the *AnmatA* genomic region (primers AnMatAF11 and AnmatAR11) into the ppyrG plasmid (Pyrzak *et al.* 2008). Deletions of the 5′-upstream regulatory region were accomplished using primers indicated in Table 2 with a site-directed mutagenesis kit (Invitrogen) and pWP3 plasmid as a substrate. The 5′ deletions *matA*(−898), *matA*(−512), *matA*(−248), and *matA*(−170) used primer AnMatAR11 plus matASE2F, matAF40, matAF31, or AnmatAF32ΔmatA, respectively (Table 2). The internal deletion (−170) used primers AnmatAF38 and AnmatAR38. Deletion constructs carrying deletions of the upstream genomic region pWP3 or internal deletions of pWP3 (Δ170 bp) were used to complement the UI465 *matA*(0) strain.

5′-fluoroorotic acid selection

A. nidulans strains with a *matA* transgene integrated via 3′ single integration at the *matA*(0) locus were used to select

rare mitotic recombination events that resulted in excision of the *matA*(0) allele as well as the *pyrG* gene and restoration of the wild-type (wt) *matA* gene structure. Selection of pyrimidine auxotrophic excisants was accomplished by a modification of the 5′-fluoroorotic acid (5′-FOA) resistance scheme described by Boeke *et al.* (1984). A total of 1×10^6 conidia per plate were spread onto 5% agar plates containing appropriately supplemented minimal media and 5′-fluoroorotic acid (0.1 mg/ml). Plates were incubated at 37° for 2 days. Usually 30–50 viable colonies were recovered and analyzed for correct excision events by Southern blot analysis.

Analysis of the genomic region of the matA locus

The *matA* locus (locus ID AN4734.3) encoding the HMG box domain mating type protein and flanking neighbor genes *sygI* (AN 4733.3) and *apc5* (AN 4735.3) in *A. nidulans* are available at the Broad Institute database: (http://www.broadinstitute.org/annotation/genome/aspergillus_terreus/MultiHome.html). Putative start and end of the *matA* transcription unit were mapped on the basis of DNA sequencing of cDNAs from a sexual cycle library.

Results

Molecular organization of the matA locus and structure of the transcript

The genetic organization of *mat* genes in homothallic fungi is unique and different between individual species (Turgeon 1998). The *A. nidulans matA* locus (AN4734.3) is located on chromosome III within a domain that includes three closely linked transcriptional units, (*sygI*, *matA*, and *apc5*) (Figure 1). *sygI* (suppressor of yeast *gpa1*) encodes a homolog of a budding yeast signal transduction modulator involved in mating. *apc5* (anaphase promoting complex) encodes a protein involved in anaphase initiation during mitosis and meiosis.

matA encodes a single transcript (1851 nt) with a short 5′-UTR (134 nt) coding region (958 nt) and a long 3′-UTR (759 nt). The transcript has three introns, two within the coding region and one in the 3′-UTR. Potential transcription start sites and end of the *matA* transcript have been identified by sequencing full-length cDNA clones. The 5′ end of the longest cDNA was used as the +1 reference for this study and is referred to as transcriptional start site 1 (TSS1) (Figure 1).

Functional complementation of the matA(0) deletion depends upon the chromosomal position of the matA transgene

We created the *matA*(0) strain UI465, which carries a complete deletion of the *matA* transcriptional unit. The entire transcription unit, including start of the transcription +1, coding region, and end of the 3′-UTR, was replaced by the *A. fumigatus* prototrophic marker *argB* (Figure 2, A and B). Deletion of *matA* abolishes sexual development without affecting vegetative growth or conidiation (Figure 3, A and B).

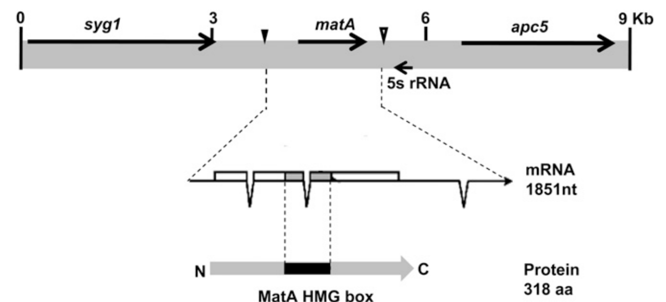


Figure 1 Genetic context of the *matA* gene on chromosome III. Black arrows indicate arrangement and orientation of genes (coding regions) flanking the *matA* locus. The *matA* transcript and protein structure are shown. Solid triangle indicates the reference transcriptional start site 1 (TSS1), the start of the transcriptional unit. Open triangle indicates the end of the *matA* transcript.

UI465 is completely sterile and does not differentiate fertile fruiting bodies. Tiny, barren cleistothecia are observed at rare frequency. An additional phenotype of the *matA*(0) deletion is a massive proliferation of Hülle cells (Figure 3B). In a wild-type *A. nidulans* strain, Hülle cells appear at the onset of sexual development and form ornamented clusters of tissue surrounding the growing, young fruiting body. Hülle cells become less prevalent when mature fruiting bodies are present. Excessive proliferation of Hülle cells in the UI465 strain might be indicative of unsuccessful fertilization events that prevent further differentiation of fruiting bodies (Figure 3B). Our *matA*(0) phenotype is similar to, but more severe than the *matA* deletion previously described by Paoletti *et al.* (2007).

The UI465 *matA*(0) strain, a *pyrG*89 auxotroph, was complemented using pWP3, which has the prototrophic marker *AnpyrG* and a wild-type copy of the *matA* transcription unit flanked by 1 kb of 5′ and 1.2 kb of 3′ genomic regions. Homologous recombination of the plasmid at *matA*(0) on chr III resulted in integration of the *matA* transgene at the resident locus, whereas homologous recombination at the *pyrG* locus on chr I resulted in the ectopic integration of the *matA* transgene (Figure 2, C and D). The ectopic *matA* transgene fully complemented the *matA*(0) allele on chromosome III (Figures 2C and 3C). The complemented *matA*(0) strain had a fertile wild-type phenotype, with normal fruiting body abundance, size, and ascospore production. Surprisingly, integration of a single copy of the *matA* transgene at the resident *matA*(0) locus only partially restored wild-type phenotype (Figures 2D and 3D). The partially complemented strain differentiated abundant cleistothecia; however, asci and ascospore formation was dramatically reduced by 80% compared to wild-type levels. The complementation phenotypes of the *matA*(0) strain are consistent with the complementation results for a partial deletion *matA*Δ strain (K. Y. Miller, W. Czaja, and B. L. Miller, unpublished data). These data suggest that the molecular function of a *matA* transgene depends on its genetic position and implies complex regulation of the resident *matA* locus.

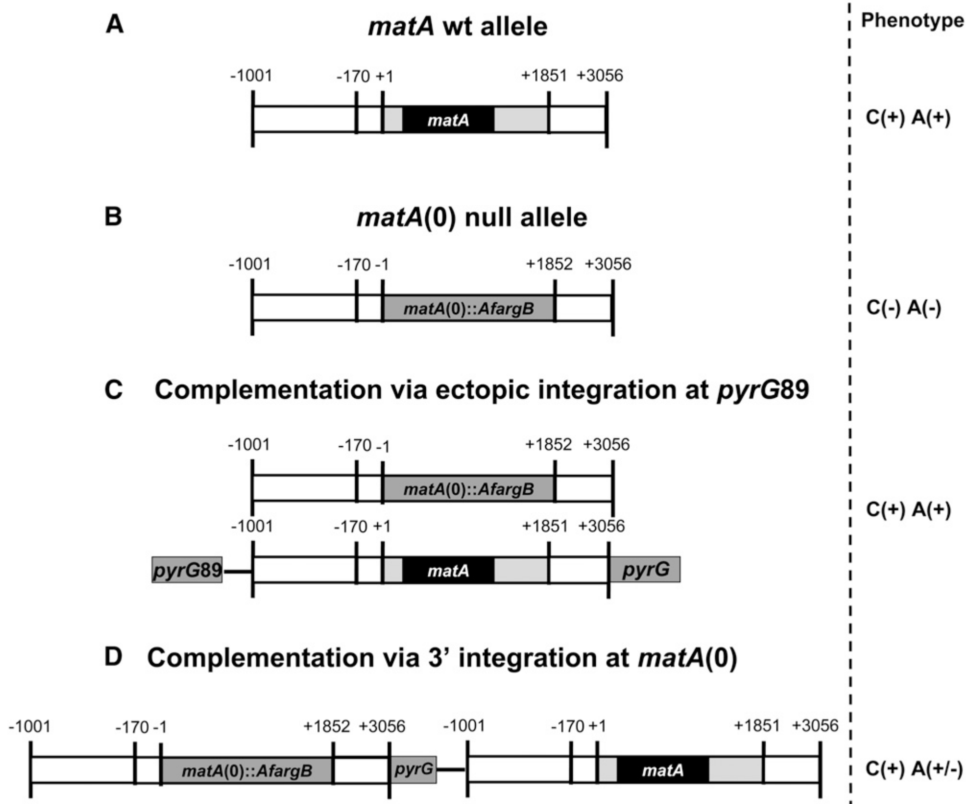


Figure 2 Schematic representation of the deletion and complementation of *matA*. (A) Wild type *matA* allele; solid bar represents coding region (ORF), lightly shaded bars represent *matA* 5′- and 3′-UTRs, and open bars represent upstream 5′ and downstream 3′ genomic sequences flanking the transcriptional unit. Horizontal solid line represents vector sequence of pWP3. Physical distance is marked (−1001 to +3056 bp). (B) Null *matA*(0) allele; the *matA* transcriptional unit was deleted and replaced with the *Aspergillus fumigatus argB* (*AfargB*) marker. (C) Complementation via ectopic integration; *matA* complementing transgene was integrated ectopically at the *pyrG*89 locus on chromosome I. (D) Complementation via 3′ integration at the *matA*(0) locus; *matA* transgene carried on pWP3 was integrated at *matA*(0) locus via homology between 3′ flanking sequences. The corresponding phenotype is shown: C, cleistothecia; A, ascospores; (+), presence; (−), absence.

Transcriptional expression of the matA transgene at the resident matA(0) locus is suppressed at late stages of sexual development

Mating type transcripts are detected on Northern blots at very low abundance. Therefore, we utilized a quantitative RT–qPCR approach to analyze expression of the *matA* transgene during sexual development. *matA* expression was analyzed in the growing mycelia (undifferentiated hyphae, 0 days post-induction, dpi) and in the reproductive tissue at 2, 4, and 6 dpi of sexual development. These developmental time points correlate, respectively, with the progressive differentiation of the fruiting body from protocleistothecia with Hülle cells, young pink-walled cleistothecia with ascogenous hyphae and mature dark purple-pigmented cleistothecia with asci

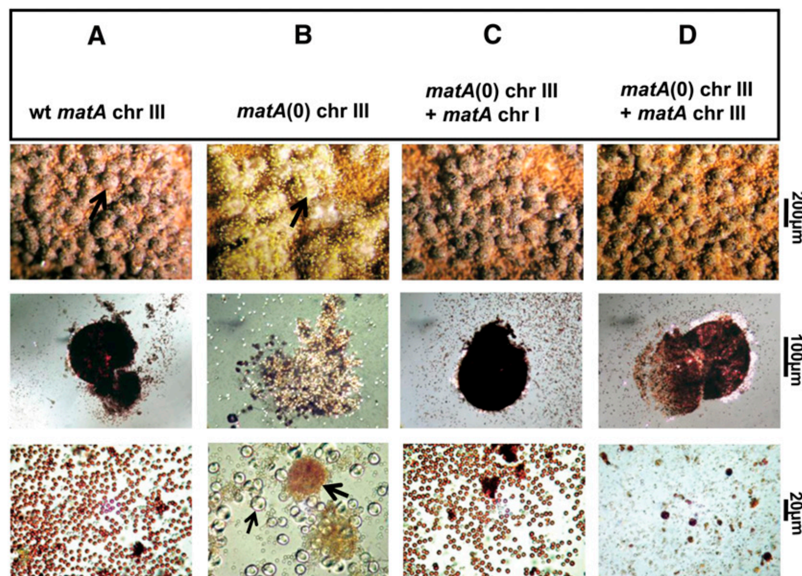


Figure 3 Sexual development in wild type (wt), *matA*(0), and complemented *A. nidulans* strains. Representative strains (A–D) were induced to undergo sexual development as described in *Materials and Methods*. Abundance of cleistothecia and internal reproductive tissue were examined under specific levels of magnification as indicated. (A, C, and D, top row) Morphology and abundance of mature cleistothecia on rich medium agar plates in wild-type and complemented strains (arrow in A indicates a single cleistothecium). (B, top row) Cluster of hülle cells in the *matA*(0) strain. (A, C, and D, middle row) Individual mature cleistothecia in wild-type and complemented strains. (B, middle row) Mass of hülle cells in the *matA*(0) strain. (A, C, and D, bottom row) Efficiency of ascospore production in wild-type and complemented strains, respectively. (B, bottom row) Sexual development in the *matA*(0) strain is aborted at the stage of unfertilized protocleistothecia, shown as brown spherical structures (thick black arrow) with individual hülle cells (thin black arrow).

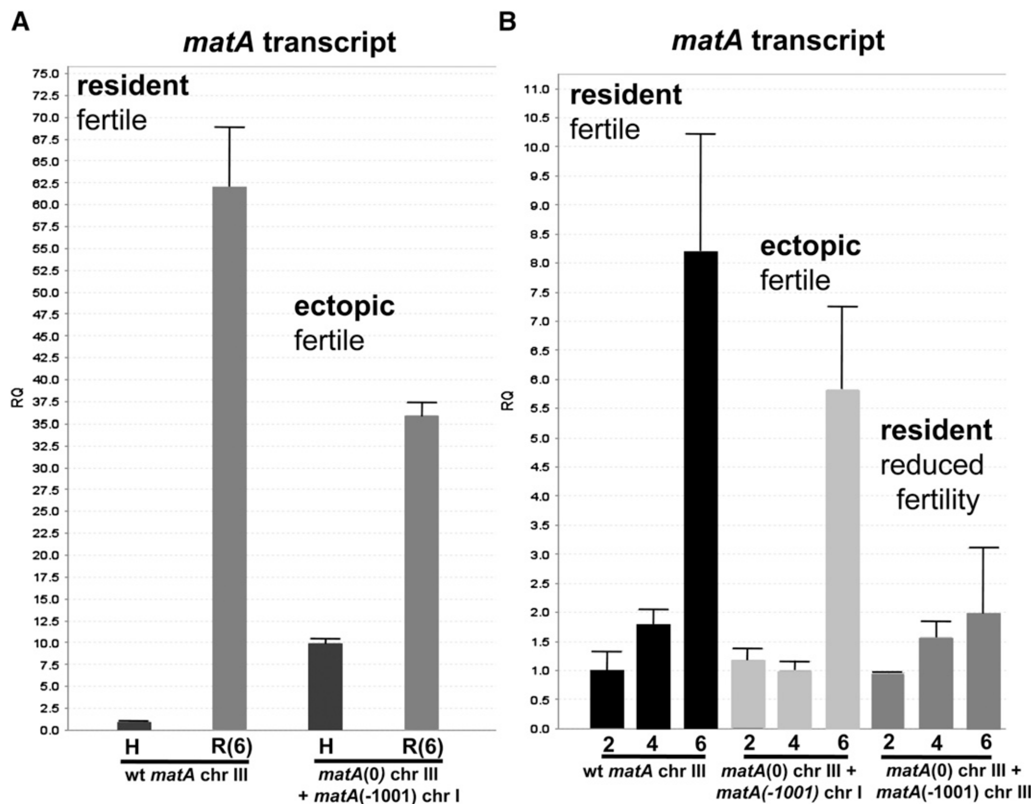


Figure 4 Comparative transcriptional expression of *matA* at resident and ectopic loci. Phenotype associated with a specific abundance of *matA* transcript in the reproductive tissue is indicated. (A) Relative quantitation (RQ) of *matA* expression from the endogenous *matA* locus on chromosome (chr) III and from the ectopically integrated *matA* transgene on chr I. The transgene carries 1000 bp of 5′ flanking upstream sequences. H, undifferentiated hyphae; (R6) reproductive tissue, 6 days postinduction. (B) Relative quantitation of *matA* expression over a developmental time course at 2, 4, and 6 days postinduction of sexual development. Expression profiles of the endogenous *matA*, ectopically integrated *matA* transgene and the *matA* transgene integrated at resident or ectopic loci are shown.

and ascospores. We first compared *matA* expression in undifferentiated hyphae (0 days) and in reproductive tissue (6 days postinduction) in a wild-type strain with a functional *matA* at the resident locus and the complemented *matA*(0) strain carrying a functional *matA* transgene integrated ectopically (Figure 4A). Very low *matA* transcript abundance transcript was detected in undifferentiated wild-type hyphae, whereas there was a dramatic 62-fold upregulation in reproductive tissue. By contrast, *matA* expression in the ectopically complemented strain was derepressed 10-fold in undifferentiated hyphae and downregulated by 30–40% in the reproductive tissue relative to wild type (Figure 4A). The difference in expression pattern of the ectopic *matA* transgene suggests that *cis*-regulatory elements and *trans*-acting factors modulate *matA* expression in both a position-dependent and position-independent manner. However, these differences in developmental *matA* expression apparently do not affect biological functions of the MatA protein. Elevated expression in hyphae did not promote precocious sexual reproduction in the ectopically complemented strain and reduced levels in developmental tissue were sufficient to drive differentiation of wild-type cleistothecia with mature ascospores (Figure 3C).

matA expression was further analyzed in reproductive tissue from different stages of fruiting body development: 2, 4, and 6 dpi of sexual cycle (Figure 4B). In the wild-type strain the highest expression of transcript is detected at 6 days postinduction, which correlates specifically with active meiotic and mitotic divisions and differentiation of mature asci with ascospores. Further progression of sexual development leads to a fully mature cleistothecium and a gradual decrease of *matA* transcript level (confirmed by Northern blot and RT–qPCR, data not shown).

The ectopically integrated *matA* transgene had a developmental expression profile (2, 4, and 6 dpi) similar to wild type, with a characteristically significant upregulation of the *matA* transcript at 6 dpi. Surprisingly, expression of the *matA* transgene integrated at the resident *matA*(0) was significantly suppressed (~25% of wild-type expression) at 6 dpi (Figure 4B). These low levels of *matA* transcript during the critical later stages of cleistothecium development are apparently insufficient and contribute to failure in ascus and ascospore development.

5′ flanking DNA sequences are dispensable for matA molecular function during sexual reproduction

We performed deletion analyses of the 5′ genomic region upstream of TSS1 to identify a putative promoter region and regulatory elements driving functional *matA* expression. The distal 830 bp of the 5′ flank (from −171 to −1001 bp) upstream of *matA* was deleted from the pWP3 vector using site-directed mutagenesis. The resulting pWP3^{-170} deletion construct was transformed into the UI465 *matA*(0) recipient strain. Transformants carrying single homologous ectopic integrations of pWP3^{-170} at *pyrG*89 were analyzed for complementation of the *matA*(0) phenotype. In all tested transformants, ectopically integrated pWP3^{-170} fully complemented resident *matA*(0) deletion and restored wild-type

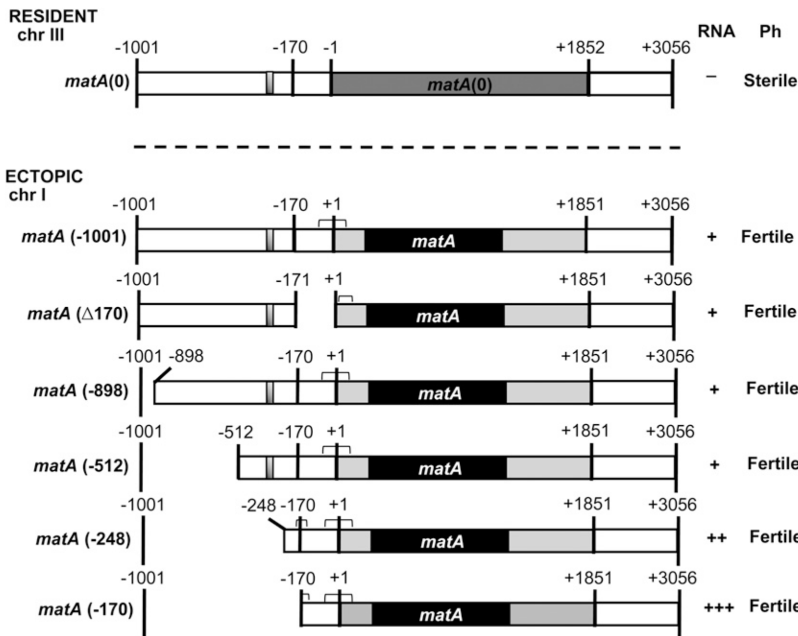


Figure 5 Deletion analysis of the 5′ flanking region of *matA*. The solid bar indicates the *matA* coding region, lightly shaded bars represent 5′- and 3′-UTRs. The silencer element is indicated as a small shaded box. Chromosomal positions in the genome are indicated (chr III and chr I). Positions of 5′ deletion end points and internal deletions of upstream flanking sequences are indicated by − and Δ, respectively. Transgene deletions were integrated ectopically at the *pyrG* locus on chr I. Complementation was observed for all transgene constructs. Transcript presence (+) or absence (−) is indicated. Phenotypes associated with each deletion are indicated. Horizontal brackets indicate positions of mRNA cap sites determined for hyphal and developmental RNAs using RACE (refer to Figure 7).

sexual development (Figure 5). Thus, sequence from −1000 to −171 is dispensable for expression levels required for proper *matA* function during fruiting body development and ascospore differentiation.

We analyzed the proximal portion of the 5′ flank (−1 to −170 bp) upstream of TSS1 to determine whether these sequences function as a promoter region. A pWP3$^{\Delta170}$ deletion construct having this internal 170-bp deletion (see *Materials and Methods*) was transformed into the UI465 *matA*(0) recipient strain. A single copy of the pWP3$^{\Delta170}$ construct integrated ectopically at *pyrG*89 fully restored the wild-type sexual phenotype (Figure 5). This observation indicates that the 5′ flanking 170 bp of genomic sequences are not essential to drive functional *matA* transgene expression. Further, no sequence duplications are detected in the −1 to −170 and −171 to −1001 regions that might suggest the possibility of redundant promoters. Taken together, these data suggest that typical promoter elements do not lie within the upstream region from −1 to −1001 bp relative to *matA* TSS1. We therefore mapped *matA* mRNA cap sites in both hyphal and developmental tissues from wild-type and deletion strains to identify potential alternate or novel promoter elements that may lie at, or downstream of TSS1 and are required to drive *matA* transcription (see below).

Deletion of the 830 bp of 5′ regulatory flank dramatically alters developmentally regulated matA expression without affecting phenotype

To determine whether 5′ flanking sequences had a role in developmentally regulated *matA* expression, complemented strains with an ectopically integrated *matA* transgene carrying the −170-bp or internal Δ170-bp deletion were analyzed. *matA* transcript abundance in the wild-type strain was upregulated ∼55-fold upon transition from undifferentiated hyphae to reproductive tissue (Figure 6A). In the UI465 *matA*(0) strain there was no detectable level of *matA* transcript (data not shown). Interestingly, the hyphal level of *matA* transcript was dramatically derepressed 135-fold in the complemented strain UI467 (−170 bp) compared to wild-type hyphal expression. Expression in reproductive tissue was also derepressed, being 2.5-fold higher relative to wild type and ∼3-fold higher relative to the ectopic control strain (Figure 6A). Therefore, the 830-bp upstream region (from −170 to −1001 bp) has a critical regulatory element/s involved in the developmental repression of *matA* gene. This dramatic increase of *matA* transcript in the hyphae and reproductive tissue did not alter sexual development. However, we cannot rule out the possibility that derepressed *matA* expression results in increased fertilization efficiency or precocious fertilization as these events are not readily detectable in homothallic *A. nidulans*. By contrast, strain UI468 carrying the 170-bp internal deletion of the proximal portion of 5′ flanking sequences, maintained an expression profile consistent with the ectopic *matA* transgene having the complete 1 kb of 5′ flanking DNA (Figure 6A). Therefore, the 170 bp proximal to TSS1 does not appear to be necessary for developmentally regulated *matA* transcription.

A putative silencer element (matA SE) and novel promoter elements regulate matA expression

Vegetative repression of *matA* in wild type and derepression in UI467 suggest that a silencer element/s is involved in developmental expression patterns. Analysis of the distal portion of 5′ flank revealed the presence of a short (29 bp) DNA segment localized between −931 and −960 bp that has striking structural similarity to the hSRY silencer (Su and Lau 1993). This 29-bp element and hSRY SE share 83% identity and 93% similarity (data not shown). However, a deletion series between

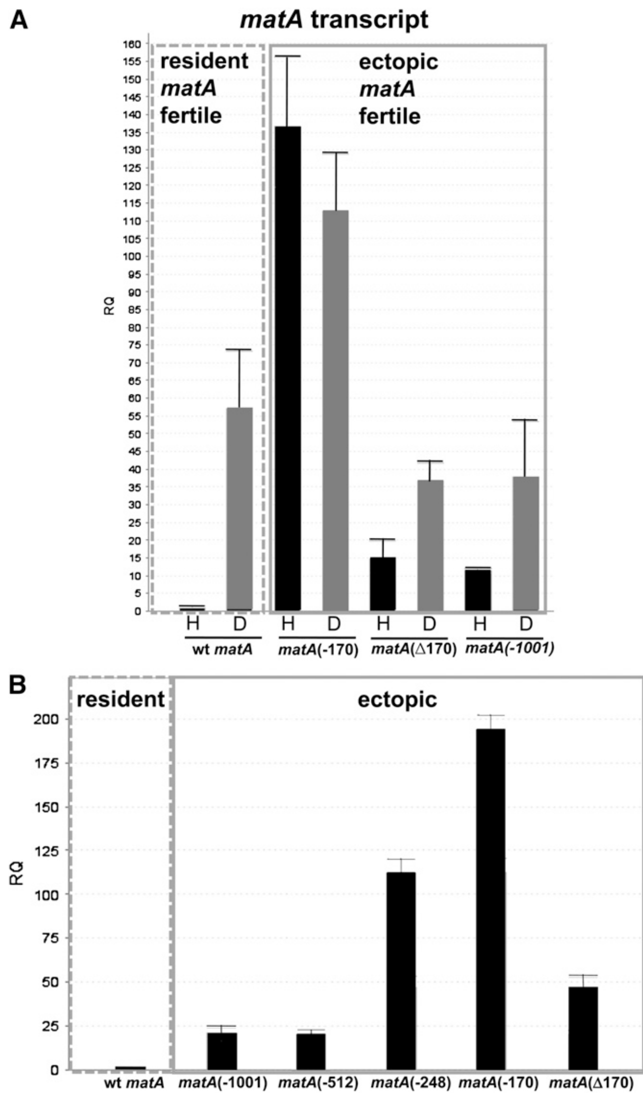


Figure 6 Comparative expression of *matA* transgene deletions. (A) Developmental expression of *matA* in transgene strains lacking either 830 bp of upstream sequence or the internal 170 bp proximal to the start of transcription. The level of *matA* transcript was analyzed in the undifferentiated hyphae, H, and in the reproductive tissue, D, at 4 days post-induction of sexual development. The dashed line box indicates expression of *matA* at the resident locus in the wild-type strain. The solid line box indicates expression of *matA* transgenes at the ectopic locus. (B) Expression in additional 5′ deletion strains used to determine position of upstream repressor and promoter elements. Specific 5′ deletion end points or internal deletions are indicated by − or Δ, respectively.

−1001 and −512 indicated that deletion of this sequence does not affect sexual development or ectopic *matA* expression under our experimental conditions (Figures 5 and 6B). By contrast, we did detect a functional *matA* SE within sequences between −512 and −170. Compared to the ectopic *matA* control strain (−1001 bp), *matA* expression in deletion strains UI465 3.5 (−248 bp) and UI467 (−170 bp) was ~5-fold and ~8.5-fold derepressed, respectively (Figure 6B). UI468 (Δ170) also shows a small ~2-fold derepression in hyphal *matA* expression (Figure 6B). Located within these sequences are four repeats having the consensus 5′-TRAARSRAARAAYYGRR-3′, which may serve as silencing elements. Two repeats are found between −248 and −512, one between −1 and −109, and a truncated repeat between −170 and −248.

We scanned the 200 bp upstream of TSS1 for canonical TATA box promoter motifs. No A/T rich or other consensus sequences resembling the TATA box were found. Pyrimidine tracts that function in some fungal promoters are also not found upstream of *matA* TSS1. There are two potential upstream CAAT elements. However, our functional analysis of the proximal 5′ flank (−1 to −170 bp) confirmed that this region does not contain essential promoter elements and was dispensable for developmental regulation of *matA*. It is possible that transcription is initiated from an initiator element (Inr) located at or near the transcriptional start site. An Inr is functionally similar to a TATA box but can act independently (Smale and Kadonaga 2003). Two putative Inr elements can be identified on the basis of DNA consensus sequences and similarity to the Inr in *Drosophila* (TCA(+1)$^{G}/_{T}$TY) and mammals (PyCA(+1)NTYY) (Smale and Kadonaga 2003) (Figure 7). One is located at TSS1 (+1) and the other at +36. *matA* mRNA cap sites were mapped in hyphal and developmental RNAs to determine functionality of these elements. Cap sites for wild-type and ectopically integrated constructs were also compared to determine whether any deletion resulted in aberrant transcriptional initiation. We found that transcription is initiated approximately equally from three zones in both hyphal and reproductive tissue (Figure 7). Zone A corresponds to the putative Inr at TSS1 while zone C corresponds to the downstream Inr at +36 relative to TSS1. However, a third distinct cluster of transcriptional start sites was centered at +18 relative to TSS1. Sequence at this position does not resemble an Inr or any other recognized promoter element. Closer inspection of sequences around the three clusters of mRNA cap sites revealed that zone A is probably not an Inr. Rather zones A and B share very similar sequences, with most cap sites centered within two overlapping repeats having the consensus 5′ YKAGACACMRTCTYCT 3′. This sequence represents a novel promoter element not previously identified. *matA* mRNA cap sites for hyphal and developmental RNAs from wild-type, ectopic control, and deletion strains all initiated from these three zones except for UI468 (Δ170), which lacked zone A. Transcription in this strain was initiated only from zones B and C. Transcription in developmental tissue from overexpression strains UI465 3.5 (−248) and UI467 (−170) was initiated from these same three zones, similar to wild type. However, we observed an additional, novel cluster of cap sites in hyphal tissues from these two strains. This cluster was centered at −169 relative to TSS1 (Figure 7). Surprisingly, this indicates that transcription in hyphae of UI467 was initiated at the very end of the deletion and without any *matA* upstream sequences. There is no sequence resembling an Inr or any other known promoter element located near −170, indicating that this may represent another novel promoter element

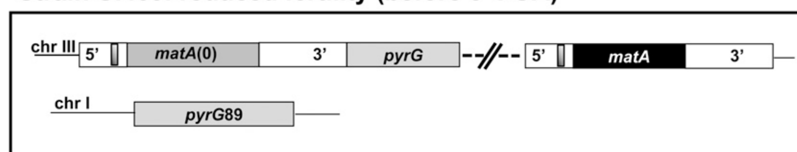
Figure 7 Identification of both Inr and novel promoter elements that regulate *matA* transcription. Mapping of mRNA cap sites identified three zones of transcriptional initiation in hyphae and developmental tissue from wild-type and deletion strains (A–C). Each zone is indicated by brackets. An asterisk marks a fourth zone near position −170 observed in hyphal tissue from derepressed strains *matA* (−248) and *matA* (−170). The position of the majority of cap sites within a zone is indicated by a thick arrow. Thin arrows mark two minor sites of mRNA start sites. The reference +1 represents the 5′ end of the largest cDNA previously identified from a sexual development library. Potential Inrs are indicated in blue; novel promoter elements are underlined. The translational start site (TSL) and beginning of the ORF are in red text. A black dot below the text marks the −170 position for reference.

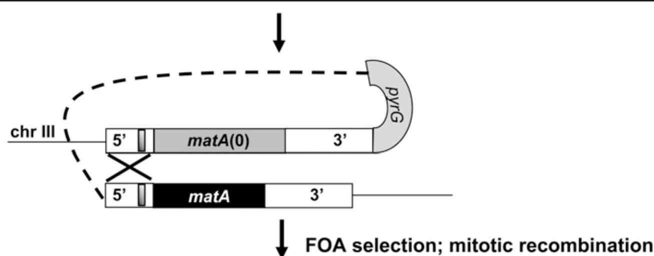
that recruits the transcriptional apparatus to this site, or that an unknown downstream promoter element directs transcriptional initiation upstream near −170. Regardless, the *matA* SE identified above normally suppresses expression from this cryptic promoter element in hyphae.

Removal of the matA(0) allele and duplicated flanking sequences from the resident matA locus restores wild-type expression of the matA transgene and wild-type phenotype of the complemented strain

Integration of a functional *matA* transgene at the *matA*(0) locus on chromosome III resulted in greatly reduced developmental transcript abundance and reduced fertility. Integration of the pWP3 plasmid with the functional *matA* transgene at the *matA*(0) locus duplicates *matA* 5′ and 3′ flanking sequences, which could potentially alter the distance and/or copy number of important regulatory elements that control functional *matA* expression. To further understand the basis of altered expression of the *matA* transgene at *matA*(0), we deleted the entire *matA*(0) allele along with its flanking 1-kb upstream and 1.2-kb downstream genomic regions. This leaves a single copy of the intact *matA* gene at the resident locus on chromosome III. This was accomplished using 5′-FOA selection to isolate mitotic recombinants that had looped out the *matA*(0) copy including the *pyrG* gene plus plasmid sequences of pWP3 (Figure 8). All recombinant strains recovered after 5′-FOA treatment had restored wild-type phenotype with abundant cleistothecia and ascospore formation. We confirmed that functional *matA* expression had also been restored in these strains by analyzing expression in undifferentiated hyphae (0 days) and 6-dpi reproductive tissue in three strains: wild type (*matA* resident copy), UI463 [*matA*(0) + *matA* transgene at resident locus], and UI463-1 (reconstructed *matA* resident copy). *matA* expression was restored to wild-type levels upon deletion of the *matA*(0) allele and its associated flanking 5′ and 3′ sequences from the resident *matA* locus (Figure 9). Therefore, we excluded the possibility that undetected secondary mutations alter sexual development. Instead, our data suggest that the physical presence of the duplicated flanking DNA sequences at the resident

Strain UI463: reduced fertility (before 5' FOA)





Strain UI463-1: fertile (after 5' FOA)

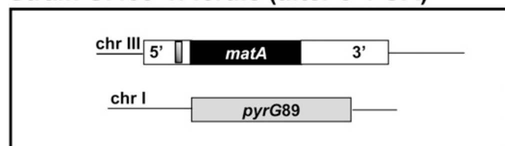


Figure 8 Schematic depiction of *matA* flanking sequence duplication and restoration of wild-type *matA* gene structure at the endogenous locus. Genetic organization of the *matA* locus after 3′ homologous integration of pWP3 carrying the *matA* transgene (solid box). 5′ and 3′ flanking regions are shown with the silencer element (shaded box). The DNA fragment containing *pyrG* and the *matA* transgene allows alignment of duplicated 5′ flanking regions and rearrangement during mitotic recombination. The *matA*(0) allele and pWP3 carrying *pyrG* are excised and wild-type *matA* allele is restored at chromosome III. Recombinant events containing wild-type *matA* and the *pyrG*89 mutation (chr I) were selected after plating on media supplemented with 5′-FOA.

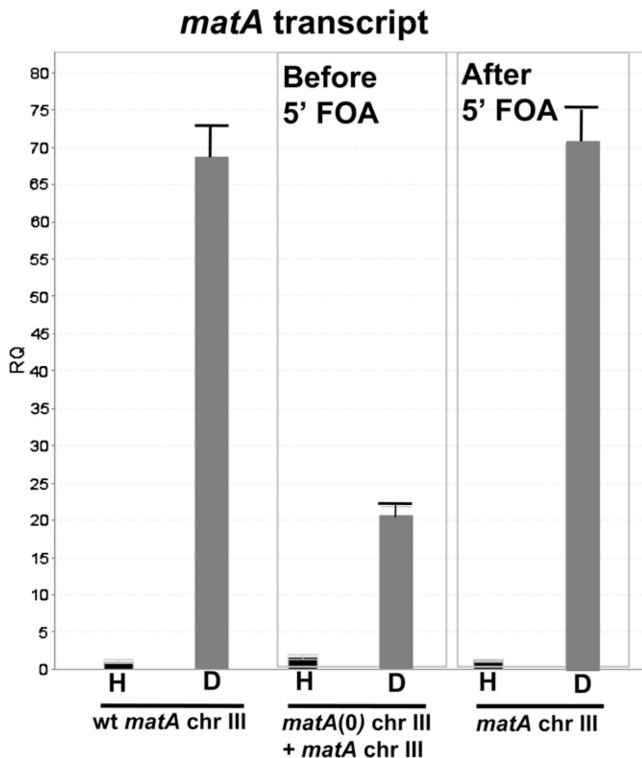


Figure 9 Developmental expression of *matA* at the resident locus. Expression of *matA* was analyzed in undifferentiated hyphae, H, and in reproductive tissue 6 days postinduction of sexual development, D. Expression of the *matA* transgene at the *matA*(0) locus is suppressed in the reproductive tissue (before 5′-FOA). Wild-type expression of *matA* is restored at the resident locus (after 5′-FOA) upon removal of *matA*(0) and plasmid sequences and the recovery of wild-type genomic structure.

mat locus interferes with the functional expression of the complementing *matA* gene.

Duplication of 5′ flanking sequences containing the matA SE interferes with the expression of the matA transgene at the resident mat locus

Partial functionality of the *matA* transgene at the resident *matA*(0) locus could be the result of duplication of the upstream and/or downstream noncoding regions that flank both *matA* transgene and *matA*(0) allele. To test this possibility, we analyzed a strain with a single integrated copy of a *matA* transgene lacking the distal −830 bp at the resident *matA*(0). This 5′ truncated transgene fully complemented the deletion and restored full development of wild-type cleistothecia with mature ascospores. Therefore, suppression of the *matA* transgene at the *matA*(0) that we previously observed is correlated with duplication of the 830 bp containing the putative *matA* SE silencer element/s described above. We also analyzed a single integration of the pWP3$^{\Delta170}$ construct at the resident *matA*(0) locus. This integrated transgene retains the *matA* SE and resulted in reduced fertility and only partial complementation of the *matA*(0) phenotype. Taken together, these results confirm that duplication of the distal 830 bp of the 5′ flanking region containing the *matA* SE interferes with the expression and function of *matA* at the resident locus (Figure 10, A and B).

Figure 10 Duplication of 830 bp of *matA* 5′ flanking sequences at the resident *matA* locus interferes with the expression and molecular function of the *matA* transgene. Structure of the complemented endogenous *matA*(0) locus and associated phenotype are shown. Bars represent *matA* with flanking regions and pyrG/pyrG89 gene. Solid line represents vector sequence of pWP3; dashed line represents deleted 5′ flanking sequences from −1001 to −171. Shaded box represents silencer element. (A) Integration of the pWP3 plasmid via 3′ homology introduces a duplication of 830 bp of 5′ flanking sequences at the resident *matA* locus and interferes with fertility. (B) Integration of the pWP3 (Δ830 bp), via 3′ homology at the resident *matA* locus results in wild-type fertility.

Discussion

The genetic organization of mating type genes at *mat* loci determines sexual compatibility and a self-fertile (homothallic) and/or cross-fertile (heterothallic) mating strategy in filamentous fungi. Heterothallic fungi have an idiomorphic organization of *mat* genes that governs recognition between mating partners and promotes obligate cross-fertility. Conversely, homothallic species usually have both HMG box and α box *mat* genes present in a haploid genome. Mechanisms of sexual identity and self-/nonself-recognition during mating are not understood in these species (Yun *et al.* 2000; Rydholm *et al.* 2007; Debuchy *et al.* 2010). However, the existence of self-/nonself-recognition mechanisms in homothallic species has been well documented in *A. nidulans* and has been termed relative heterothallism (Pontecorvo *et al.* 1953; Hoffmann *et al.* 2001). Several lines of study suggest that sexual recognition in a homothallic system might be achieved by complex developmental regulation and differential expression of mating type genes. We have analyzed the developmental regulation and molecular function of *A. nidulans matA* to elucidate the molecular basis of sexual identity in a homothallic mating system.

Position-dependent and independent mechanisms control matA expression and function

A strain with *matA* deleted from chromosome III, *matA*(0), was unable to initiate a sexual cycle resulting in the absence of fruiting bodies. This self-sterile phenotype is consistent with previous studies in *A. nidulans* (Paoletti *et al.* 2007) and deletion of the *mat-HMG* gene in other homothallic fungi such as *Gymnoderma zea* and *Sordaria macrospora* (Lee *et al.* 2003; Desjardins *et al.* 2004; Poggeler *et al.* 2006).

Complementation studies revealed that *matA* expression is dependent upon its chromosomal position and suggest that the resident *mat* locus has complex regulatory mechanisms. The *matA*(0) deletion is fully complemented by a *matA* transgene ectopically integrated on chromosome I. Surprisingly, the same *matA* transgene integrated homologously at the resident *matA*(0) locus resulted in only a partial complementation phenotype with a dramatic 80% decrease in ascospore numbers relative to wild type.

Transcription of the resident *matA* gene in the wild-type strain is tightly regulated developmentally, being highly suppressed in undifferentiated hyphae and progressively upregulated in reproductive tissue. This observation indicates that MatA protein is essential throughout sexual development, but is dispensable for vegetative growth and conidiation. This is consistent with the *matA*(0) phenotype. Developmental expression of *A. nidulans matA* reported here confirms earlier observations (Paoletti *et al.* 2007; Pyrzak *et al.* 2008). Similarly, tight genetic regulation of mating type genes has been proposed for heterothallic fungi (Leubner-Metzger *et al.* 1997; Coppin and Debuchy 2000).

By contrast, developmental expression of the ectopically integrated *matA* transgene is less tightly regulated, being derepressed in undifferentiated hyphae and less upregulated in reproductive tissue. Therefore, we propose that regulated *matA* expression includes regional, position-dependent *cis* elements, that may influence chromatin organization and that lie outside the *matA* locus.

Interestingly, the altered expression profile of the ectopic *matA* transgene, with elevated vegetative mRNA abundance, did not have an observable effect on the sexual development program. However, early fertilization events in *A. nidulans* are difficult to observe and we cannot rule out an effect upon precocious development or altered mating efficiency. Transcript abundance in the reproductive tissue, though less than at the resident locus, was sufficient to drive sexual development. Apparently, this level of expression meets the critical threshold level that we have previously shown to be required to complete a fertile sexual cycle (Pyrzak *et al.* 2008).

Remarkably, expression of the same *matA* transgene integrated at the resident *matA*(0) locus was significantly reduced during later stages of sexual development. The reduced level of *matA* transcript was strongly correlated with a dramatic reduction in ascospore formation, but without an impact upon fruiting body differentiation. To our knowledge this is the first example, where the presence of a *matA*(0) deletion at the resident *mat* locus "interferes" with the functional expression of an adjacent complementing transgene. This observation suggests *mat* locus-specific, position-dependent requirements regarding distance and/or dosage of regulatory elements involved in developmental *matA* expression. It is possible that interference is due to the presence of duplicated copies of the *matA* SE silencing element when the intact *matA* transgene is integrated at the resident locus, and the ability of *trans* factors at this silencer to act at distances of at least 9–10 kb. This is supported by two observations. 5′-FOA eviction and deletion of duplicated sequences having *matA* SE restored wild-type gene structure and wild-type ascospore numbers. Integration of a *matA* transgene lacking *matA* SE at the matA(0) locus also fully complemented the deletion and restored a wild-type phenotype.

Upstream silencing elements and novel promoter elements regulate position-independent matA expression

Neither the proximal region (−1 to −170 bp) nor distal region (−171 to −1001 bp) were required for full complementation by the *matA* transgene. Sequence analysis of this region did not reveal the presence of potentially redundant promoter structure or typical TATA-like promoter elements. Two CAAT-like elements located within the proximal 170 bp can be deleted without effect. Taken together, these results suggest that *matA* transcription does not require upstream promoter elements for proper initiation, but is initiated directly from three closely spaced promoter elements located at the major starts of transcription. The most downstream element resembles an Inr, on the basis of core sequence conservation and homology to promoter elements described in *Drosophila* and mammals. More importantly, the two upstream elements represent novel promoter elements not previously described. These elements may represent specialized promoter sequences that presumably would be bound by TFIID-like complexes containing either a sexual cycle-specific TATA-binding protein-associated factor (TAF) or a sexual cycle-specific TATA-binding protein-related factor (TRF). This would be analogous to cell- and tissue-specific gene expression described in animals (Levine and Tjian 2003). Our results provide a molecular basis for the observation of Wirsel *et al.* (1998) that truncation of the 5′ noncoding region of *Cochliobolus heterostrophus MAT1-2-1 HMG*, including the major transcriptional start site, resulted in normal formation of pseudothecia and ascospores. Interestingly, studies of promoter activity for the porcine sex-determining gene, *SRY*(HMG-box), revealed that sequences located downstream of the transcriptional start site were important for the promoter function. (Pilon *et al.* 2003). Therefore, it is intriguing that the structure and regulation of the *matA* gene may be a conserved feature of sex-determining genes.

Although 5′ flanking sequences are not required for *matA* transcriptional initiation, we observed that deletion of sequences between −512 and −170 resulted in dramatic overexpression of *matA* in the hyphae and reproductive tissue compared to wild type. Several repeated elements are located in this region that may function as a silencer of *matA* expression. The *matA* SE element together with associated *trans* factor(s) represent a new master regulator of *matA* expression. A related observation has been made for the heterothallic fungus *Podospora anserina*. Repression of *SMR2(HMG)* transcription during the vegetative phase requires an upstream *cis*-acting element located between

1.4 and 4.7 kb upstream of the *SMR2* translational start (Coppin and Debuchy 2000). Analysis of the DNA sequence in the distal region of 5′ flank (from −1001 to −811 bp) revealed a conserved element similar to the mammalian MHC class silencer and with striking structural similarity to the silencer identified upstream of hSRY (Weissman and Singer 1991a; Su and Lau 1993). In line with our studies, intriguing parallels in the negative regulation governing yeast mating type genes and genes of the mammalian major histocompatibility complex have been previously reported (Weissman and Singer 1991a,b). Structural and functional similarity between regulatory elements of fungal and human origin might reflect conservation of regulatory pathways during sexual reproduction.

Elimination of developmental suppression and overexpression of *matA* did not have an observable impact upon fruiting body morphogenesis or ascospore formation. This result suggests that developmental repression of the *matA* gene may modulate some aspects of the sexual cycle but is not essential for MatA functions that are directly required for the basic process of sexual differentiation. Coppin and Debuchy (2000) also found that overexpression of *SMR2* and its derepression in vegetative hyphae, when driven by the constitutive *gpd* promoter, did not impact sexual reproduction in *P. anserina*. However, this does raise questions as to why *mat-HMG* transcription is so highly regulated and to what extent other posttranscriptional mechanisms might play a role in *matA* expression. Analyses of mating type genes in *C. heterostrophus* and *S. macrospora* indicate that *mat* genes are both transcriptionally and post-transcriptionally regulated (Leubner-Metzger *et al.* 1997; Poggeler and Kuck 2000). In general, elevated levels of *matA* transcript were correlated with higher abundance of cleistothecia and better overall efficiency of sexual reproduction in *A. nidulans*, whereas low levels during later developmental stages interfered specifically with asci and ascospore development. These data are consistent with our earlier studies (Pyrzak *et al.* 2008) and suggest that critical spatiotemporal thresholds of *matA* transcript must be met to drive meiosis and ascospore formation. Similar requirements for *mat* expression have been proposed for *P. anserina* (Debuchy 1999; Coppin and Debuchy 2000).

Concluding remarks

Our studies provide novel insights into mechanisms underlying the regulation and molecular functions of the *matA* gene, an essential modulator of sexual processes in homothallic *A. nidulans*. Collectively, our data demonstrate that intricate mechanisms govern functional expression of this HMG-box mating type gene during self-fertile reproduction. Analyses of the developmental pattern of *matA* expression reveals unique and distinctive features, but also shows parallels to mating systems in other fungi and, intriguingly, to certain aspects of the regulation of mammalian sex determination. Manipulation of the *matA* regulatory region resulted in abundant expression of *matA* in both vegetative hyphae and sexual tissue, which resembles the constitutive expression pattern of mating type genes in some heterothallic fungi such as *N. crassa* and *P. anserina* (Ferreira *et al.* 1998; Coppin and Debuchy 2000). Therefore, it is tempting to speculate that conversion between homothallic and relative heterothallic reproductive mode in *A. nidulans* could be accomplished by changes in the expression pattern of *mat* genes. Molecular dissection of the regulation of mating type genes may also provide important insights into the genetic basis and molecular mechanisms that control sex determination and sex chromosome evolution in eukaryotes. Further studies of the developmental regulation of *matA* and other fungal mating type genes should provide significant insights into the origin, evolution, and choice of homothallic and heterothallic reproductive lifestyles, particularly in those species capable of both self-fertility and cross-fertility.

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