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The novel *Aspergillus fumigatus MAT1-2-4* mating-type gene is required for mating and cleistothecia formation

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ABSTRACT

Sexual propagation accompanied by recombination and the formation of spore-containing fruiting bodies is a cornerstone of fungal genetics and biology. In the human pathogen *Aspergillus fumigatus* sexual identity has previously been shown to be determined by *MAT1-1-1* or *MAT1-2-1* genes which act as transcriptional regulators and are present within idiomorphs found at the *MAT* locus. We here report the identification and first characterization of a further novel gene, termed *MAT1-2-4*, that is present in the *MAT1-2* idiomorph of *A. fumigatus*. A mating-type swapping strategy was used to achieve an unbiased deletion of the *MAT1-2-4* gene with no impact on *MAT1-2-1* gene expression. Phenotypical characterization of the resulting strain revealed an inability to mate with the compatible *MAT1-1* progenitor, demonstrating that the *MAT1-2-4* gene product is a genuine mating-type factor required for correct sexual development. A GPI-anchored protein of unknown function was identified as interaction partner. However, no functional role in the mating process or ascosporogenesis could be demonstrated by deletion analysis for this latter protein, although a role in heterokaryon formation is suggested. Bioinformatic analysis also demonstrated the presence of *MAT1-2-4* homologues in some, but not all, other *Aspergillus* species and the evolutionary origins and implications of the *MAT1-2-4* gene are discussed.

1. Introduction

The sexual identity of Eukarya is determined at the genetic level by specialized elements such as sex chromosomes or loci, with conservation of some features seen across kingdoms (Fraser et al., 2004). In fungi, so called 'mating-type (*MAT*)' systems have evolved to facilitate recombination between genetically distinct isolates. The genetic basis of mating-type has been thoroughly studied in the Dikarya fungal subkingdom leading to the identification of two distinct systems. The Basidiomycota have been found to have evolved a tetrapolar system with multiple mating-types expressed from two unlinked *MAT* loci that need to differ for sexual compatibility in outcrossing species, while a bipolar mating system is present in most Ascomycota, made up of two mating-types that determine sexual compatibility. In the course of fungal sexual interaction, several pivotal stages have to be undergone. There is the initial requirement for detection and recognition of a compatible partner, followed by mating with accompanying cellular and nuclear fusion events, and eventually the formation of sexual spores which are harbored within protective fruiting bodies. All of these processes are determined by a plethora of cellular components, such as receptors and respective pheromones, signal transduction cascades, and regulatory factors, which are orchestrated according to mating-type. Thus, genes expressed from the *MAT* loci are generally viewed as to encode master regulators of sexual propagation, and fungal mating-type systems have served as instructive paradigms for this fundamental eukaryotic trait (Dyer and O'Gorman, 2012). Besides its importance for reproduction, mating and sexual propagation have been demonstrated to be relevant for a variety of fundamental traits influenced by genetic recombination, such as fitness, virulence, and resistance (Klix et al., 2010).

In filamentous ascomycetes (the subdivision Pezizomycotina), several *MAT* loci from various fungal genera have been characterized revealing a common and conserved pattern of genetic architecture with certain basic components. In obligate outcrossing 'heterothallic' species a single *MAT* locus is normally present in complementary *MAT1-1* and *MAT1-2* isolates. This *MAT* locus is present at the same homologous chromosomal location in isolates of different mating-type, but whereas the neighbouring regions are highly conserved in sequence, the *MAT*

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Table 1
A. fumigatus strains used in this study.

Strain	Progenitor	Description/genotype	Reference
D141		Wild-type clinical isolate (*MAT1-1*, syn. NRRL 6585)	Reichard et al. (1990)
Af293		Wild-type clinical isolate (*MAT1-2*, genome sequence reference strain)	Nierman et al. (2005)
AfS35	D141	*akuA::loxP, MAT1-1*	Krappmann et al. (2006) and Wagener et al. (2008)
AfS169	AfS35	*akuA::loxP, mat1-1::MAT1-2, MAT1-2-4 ⟨six⟩*	This study
AfS170	AfS35	*akuA::loxP, mat1-1::MAT1-2, MAT1-2-4Δ ⟨six⟩*	This study
AfS202	AfS35	*akuA::loxP, MAT1-1, miaA::six*	This study
AfS203	AfS169	*akuA::loxP, mat1-1::MAT1-2, MAT1-2-4 ⟨six⟩, miaA::six*	This study
AfS204	D141	^{P}*niiA::N-yfp::MAT1-2-4::niiA*^{t}-^{P}*niaD::yfp-C::miaA::niaD*^{t}, *ptrA*	this study
AfS205	D141	^{P}*niiA::N-yfp::MAT1-2-4::niiA*^{t}-^{P}*niaD::yfp-C::miaA*$^{GPI\Delta}$*::niaD*^{t}, *ptrA*	This study
AfS206	AfS170	reconstituted *MAT1-2-4* strain	This study
AfS207	Af293	^{P}*niiA-niaD::MAT1-2-4::gfp2-5::his2A*^{t}, *ptrA*	This study
AfS223	AfS35	*akuA::loxP; MAT1-1;* ^{P}*gpdA::mCherry::his2A ⟨ptrA⟩*	This study
AfS224	AfS170	*akuA::loxP, mat1-1::MAT1-2, MAT1-2-4Δ ⟨six⟩;* ^{P}*gpdA::gfp2-5::his2A ⟨ptrA⟩*	This study
AfS225	AfS206	*akuA::loxP, mat1-1::MAT1-2, MAT1-2-4 ⟨six⟩;* ^{P}*gpdA::gfp2-5::his2A ⟨ptrA⟩*	This study

loci are highly divergent in sequence and therefore genetic information, and have been coined 'idiomorphs' to distinguish these from alleles (Debuchy and Turgeon, 2006; Dyer et al., 2016; Metzenberg and Glass, 1990). In the simplest form the *MAT* loci contain only one gene, with cell and nuclear mating identity being conferred by either a *MAT1-1-1* α-box domain or a *MAT1-2-1* high-mobility group (HMG) encoding gene in *MAT1-1* or *MAT1-2* isolates, respectively. Numerous experimental studies have confirmed the key functional importance of these *MAT1-1-1* and *MAT1-2-1* genes (Dyer et al., 2016), which have recently been shown to influence transcription of a large number of genes including several genes not directly involved in the mating process (Becker et al., 2015; Bidard et al., 2011; Böhm et al., 2013; Wada et al., 2012). However, in certain taxonomic groupings of the Pezizomycotina, two or more additional mating-type specific genes have also been found to be located at either the *MAT1-1* or *MAT1-2* idiomorphs. These additional genes have proved to be of great interest, firstly from an evolutionary perspective because they provide insights into the evolution of *MAT* regions in the Eukarya in general. Secondly, experimental work indicates that these additional genes are often (but not always) required for correct sexual development, functioning alongside the main *MAT1-1-1* or *MAT1-2-1* genes (Dyer et al., 2016). A nomenclature system for naming of mating-type genes was proposed by Turgeon and Yoder (2000), but some confusion has arisen over recent years as some genes with little evolutionary relationship have been given the same epithets (Dyer et al., 2016). This has recently lead Wilken et al. (in press) to propose a revised nomenclature system to more accurately describe the relationship of *MAT* genes.

In the genus *Aspergillus*, mating and development of fruit bodies, known as cleistothecia, may occur in a homo- or heterothallic fashion by self-fertile or self-sterile species, respectively (Dyer, 2007). For the latter, the mating-type identity is encoded by a *MAT* locus that specifies either *MAT1-1* or *MAT1-2* genotype as in other heterothallic Pezizomycotina. Inspection of genomic sequences from several species has shed light on the general outline of the *MAT* loci in the aspergilli (de Vries et al., 2017). The comparison of homo- and heterothallic species has furthermore allowed speculation on the evolutionary origins of the respective breeding systems, implying that heterothallism might be the more ancestral form of sexual propagation for this genus, an assumption that nevertheless remains controversial (Dyer, 2007; Galagan et al., 2005; Lee et al., 2010).

The *MAT1-1* and *MAT1-2* mating-type idiomorphs of heterothallic aspergilli are generally relatively simple in their organization, only containing one *MAT* gene that encodes either the *MAT1-1-1* associated α-box protein or the *MAT1-2-1* associated high mobility group regulatory factor, respectively (Dyer and O'Gorman, 2012). A limited number of studies have characterized these *MAT1-1-1* and *MAT1-2-1* encoded putative transcription factors in both homothallic and heterothallic aspergilli and demonstrated their functional importance in sexual reproduction (Pyrzak et al., 2008; Große and Krappmann, 2008; Szewczyk and Krappmann, 2010). However, there is accumulating evidence that certain *Aspergillus* species might also contain additional *MAT* genes within the *MAT1-2* idiomorph as has been suggested notably for the opportunistic human pathogenic species *A. fumigatus*. In this species the identification of mating-type idiomorphs was a prerequisite for discovery of its long-time undisclosed sexuality (O'Gorman et al., 2009; Paoletti et al., 2005; Pöggeler, 2002). Cleistothecia formation in this ascomycete appears to be restricted to specific environmental conditions that support mating and eventual ascosporogenesis of compatible isolates under prolonged periods of confrontation. This fastidious behavior has been partly alleviated by the discovery of pairs of so-called 'super-maters' which form fruiting bodies in a shorter time period, making the sexual cycle of *A. fumigatus* (teleomorph *Neosartorya fumigata*) more accessible for *in vitro* studies (Sugui et al., 2011).

In previous works, the main *MAT1-1-1* and *MAT1-2-1* encoded putative transcription factors of *A. fumigatus* have been functionally characterized (Pyrzak et al., 2008; Große and Krappmann, 2008; Szewczyk and Krappmann, 2010). However, an additional putative open reading frame (ORF) with the hypothetical capacity to encode a so-far uncharacterized gene product has also been annotated in the *MAT1-2* locus (Nierman et al., 2005). A similar putative ORF has also been reported from the closely related species *N. fischeri* and *A. lentulus* (Fedorova et al., 2008; Swilaiman et al., 2013) but no further in-depth inspection of the *A. fumigatus* mating-type idiomorphs and any associated components has been pursued. In this study, first efforts are made to characterize this putative mating-type gene, termed *MAT1-2-4*, to determine whether it has functional activity. It is demonstrated that this gene encodes a genuine and novel mating factor of *A. fumigatus*, and that homologues can be found in a number of other *Aspergillus* species.

2. Materials and methods

2.1. Strains, media, and growth conditions

Aspergillus fumigatus strains used in this study are listed in Table 1. The clinical isolates D141 (Reichard et al., 1990) and Af293 (Nierman et al., 2005), as well as the *akuA::loxP* strain AfS35 (Krappmann et al., 2006; Wagener et al., 2008) served as progenitors for recombinant strains. Minimal medium (0.52 g/l KCl, 0.52 g/l $MgSO_4$, 1.52 g/l KH_2PO_4, 0.1% trace element solution, pH 6.5 [Scott and Käfer, 1982]) was used for growth of these strains, supplemented with appropriate amount of pyrithiamine (0.05 μg/ml) or hygromycin B (200 μg/ml); 1% glucose or 4% xylose was used as carbon source with either 5 mM ammonium tartrate or 10 mM sodium nitrate serving as nitrogen source; for solid medium, 2% agar was added. All strains were grown at 37 °C, with shaking at 150 rpm for liquid culture. BD Difco Oatmeal