The master regulator MAT1-1-1 of fungal mating binds to its targets via a conserved motif in the human pathogen Aspergillus fumigatus

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Abstract

Mating-type transcription factors are master regulators of sexually related signal transduction pathways in fungi; however, their recognition of specific DNA sequences from target genes is widely undetermined. Here, we identified and characterized the DNA-binding sequence of the MAT1-1-1 alpha-box domain transcription factor from the human pathogen Aspergillus fumigatus. In order to explore MAT1-1-1 DNA-binding targets, we used the previously reported MAT1-1-1 binding motif from Penicillium chrysogenum, in a bioinformatics approach. We identified 18 A. fumigatus genes carrying the MAT1.1 sequence in their upstream region, among them genes for the a-pheromone precursor (PpgA), G-protein-coupled pheromone receptor (PreA), and for TomA, an unidentified protein. To validate our prediction further, quantification of transcript levels showed a decrease in expression of ppgA, tomA, and others in a MAT1-1 deletion strain. For a functional analysis of the binding sites, truncated variants of the A. fumigatus MAT1-1-1 gene were introduced into Escherichia coli for heterologous expression. The yield of recombinant protein was further optimized for the AfMAT1-1-178–235 variant that harbors an extended alpha-box domain. AfMAT1-1-178–235 bound to a subset of the most strongly upregulated genes: ppgA, preA, and tomA. The DNA-binding specificity was confirmed by testing mutated binding sequences, as well as performing competition experiments with specific and non-specific sequences. Finally, equilibrium dissociation constants of 1.83 ± 0.26 μM were determined for AfMAT1-1-178–235 and fusion protein GST-AfMAT1-1-178–235. Collectively, these findings provide further insights into AfMAT1-1-1-mediated gene expression and imply that alpha-box domain regulators from other members of Eurotiales control fungal development in a conserved manner.

Keywords: mating type; transcription factor; DNA-binding motif; Escherichia coli protein synthesis; human pathogen Aspergillus fumigatus

Introduction

Sexual reproduction is a complex and ubiquitous process that occurs in eukaryotic organisms (Goodenough and Heitman 2014; Speijer et al. 2015). Despite the huge diversity of sexual programs found in fungi, mating-type (MAT) loci are a common feature for all fungal sections (Fraser and Heitman 2004; Idnurm et al. 2008; Coelho et al. 2017). The loci are called idiomorphs to indicate that they do not represent alleles of a single gene, despite their identical genomic localization (Metzenberg and Glass 1990). In Euascomycetes, the number of MAT genes varies and they usually code for transcription factors (Debuchy and Turgeon 2006). While members of the Sordariomycetes have rather complex MAT loci carrying several genes, MAT loci in members of the Eurotiales are simply organized and often contain a single functional reading frame for either a high mobility group (HMG) or an alpha-box DNA-binding domain transcription factor (Pöggeler 2007; Klix et al. 2010; Debuchy et al. 2014; Dyer and Kück 2017).

Belonging to the group of Eurotiales, the filamentous ascomycete Aspergillus fumigatus is a human pathogenic mold, mostly associated with invasive aspergillosis in immunocompromised patients (Fraser 1993; Hohl and Feldmesser 2007; Latgé and Chamilo 2019). Aspergillus fumigatus was considered to be an obligate asexual organism for many years, until genomic analysis revealed the presence of genes essential for sexual reproduction (Pöggeler 2002; Varga 2003; Paolletti et al. 2005). Aspergillus fumigatus is considered to be a heterothallic (self-sterile) fungus, therefore the mating process starts with the recognition between two compatible strains carrying MAT1-1 and MAT1-2 idiomorphs (Paolletti et al. 2007; O’Gorman et al. 2009). Recognition is initiated by α-type (PpgA) or α-type pheromones binding to the cognate G-protein-coupled pheromone receptors (PreA and PreB). Signal transduction activates a highly conserved mitogen-activated protein kinase pathway which triggers the transcriptional activation of proteins required for the cellular response (Elion 2000; Pöggeler 2002; Dyer et al. 2003; Seo et al. 2004). The expression of an α-type
pheromone precursor PpgA in *A. fumigatus* is under the transcriptional control of MAT proteins (Szewczyk and Krappmann 2010; Yu et al. 2018). Recently, the MAT1-2 idiomorph was found to encode an additional transcript, MAT1-2-4, whose deletion resulted in impaired fruiting body formation (Yu et al. 2017). The complete and functional sexual cycle with formation of fruiting bodies (cleistothecia) and sexual spores can be induced under laboratory conditions (O’Gorman et al. 2009; Szewczyk and Krappmann 2010; Sugui et al. 2011; Ashton and Dyer 2019). Thus, *A. fumigatus* proved to be a suitable model for investigating a reproduction system requiring two compatible partners for sexual mating in Ascomycetes.

MAT proteins from filamentous ascomycetes have been demonstrated to function beyond their previously understood role in sexual development (Wada et al. 2012; Böhm et al. 2013; Becker et al. 2015; Kim et al. 2015; Mahmoudjanlou et al. 2020). Notably, despite their different lifestyles, MAT1-1-1 orthologs from *A. fumigatus* and *Penicillium chrysogenum* have also been shown to regulate a similar number of transcriptional targets; however, among those target genes, most of them were obviously not directly involved in mating (Becker et al. 2015; Yu et al. 2018). For instance, it was shown that both transcription factors have an impact on secondary metabolite production (Böhm et al. 2013; Yu et al. 2018). Furthermore, population studies of *A. fumigatus* strains revealed an association between MAT1-1 isolates and increased virulence of those isolates (Alvarez-Perez et al. 2009; Cheema and Christians 2011). However, in another study, a pair of isogenic strains with opposite mating-type loci showed similar pathogenicity (Losada et al. 2015). The expression of genes for several secondary metabolites is mating-type dependent in *A. fumigatus* (Yu et al. 2018), thus sexual identity might have a distinct, albeit indirect effect on virulence. Epipolythiodioxopiperazine gliotoxin serves as a prominent example of a secondary metabolite that affects the virulence of *A. fumigatus* in a context-dependent manner (Kwon-Chung and Sugui 2009). In contrast, others, such as pseurotin or fumagillin, whose expression is specifically regulated by MAT1-encoded transcriptional regulators, have so far shown no contribution to pathogenicity. Further evidence for an overlap of fungal secondary metabolism and virulence arises from studies on the global regulator LaeA (Sugui et al. 2007).

Although MAT transcription factors have been functionally explored over the past two decades, relatively few studies have answered the question of how MAT transcription factors regulate expression of their downstream genomic targets, thus the protein–DNA interactions of MAT transcription factors remain poorly understood. Recently, a MAT1-1-driven transcriptional analysis in *P. chrysogenum* and *A. fumigatus* demonstrated that the MAT1-1-1 orthologs from these two fungi share a significant number of common target genes (Böhm et al. 2015; Becker et al. 2015; Yu et al. 2018). However, the specific DNA-binding sites of the *A. fumigatus* MAT1-1-1 transcription factor remain to be determined.

The alpha-box domains and HMG domains of MAT proteins from ascomycetes exhibit similar functional and structural residues, therefore they might bind to DNA in a similar fashion (Jackson et al. 2013). Since eukaryotic HMG domains were shown to be sufficient for DNA binding (Schlierf 2002; Jauch et al. 2012), we hypothesized that the region of *A*MAT1-1-1 encoding an alpha-box DNA-binding domain would be essential for the site-specific interaction with DNA.

In this study, we performed a detailed analysis of the *A*MAT1-1-1 binding to the highly conserved DNA-binding motif which is enriched in genes that were shown to be regulated by *A*MAT1-1-1 (Yu et al. 2018) or are known to be involved in sexual reproduction in aspergilli (Dyer and O’Gorman 2012). We demonstrate that the extended alpha-box domain of *A*MAT1-1-1 is sufficient for the sequence-specific DNA recognition. Furthermore, the regulatory binding sites of MAT1-1-1 orthologs from *A. fumigatus* and *P. chrysogenum* are highly conserved, suggesting common mechanisms for MAT driven gene regulation within members of the Eurotiales.

**Materials and methods**

**Transcriptional profiling by RT-qPCR**

Transcript levels of selected candidates were quantified in the MAT1-1 wild-type isolate D141 and its congenic mat1-1A deletion mutant (Szewczyk and Krappmann 2010) by real-time quantitative PCR (RT-qPCR). Total RNA serving as input was extracted from ground mycelia of three biological replicates using TRI Reagent (Sigma-Aldrich, St. Louis, Missouri) and peqGOLD Phase Trap reaction tubes (PEQLAB, Erlangen, Germany). Adequate quality was checked on a NanoDrop UV-spectrophotometer to further analyze only samples showing A260/A280 values greater than 1.8. Purification including DNase digestion was then performed with filters of the innuPREP Plant RNA Kit (Analytic Jena, Germany). Reverse transcription was carried out with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems without RNase inhibitor using 1 μg RNA as input for reverse transcription by the MultiScribe™ reverse transcriptase and following the recommended incubation protocol of 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and storage at 4°C. The resulting cDNA samples served as qPCR templates in a VIIa 7 real-time PCR system equipped with a 384-well block module (Applied Biosystems, Foster City, CA, USA). All reactions were performed in MicroAmp optical 384-well reaction plates covered with qPCR adhesive seal sheets (4titude) in a standard volume of 8 μl for each individual reaction containing 25 ng cDNA as template, primers at 800 nM concentration each, and 4 μl of the SYBR™ Select Master Mix. Pairs of oligonucleotides used for quantification of ppgA (AFUB_072280), tomA (AFUB_063230), cp9 (AFUB_064360), and AFUB_070880 transcript levels are presented in Supplementary Table S1. Reactions were performed in technical triplicates, and equilibrium dissociation curves were plotted to determine specificity of the PCR runs. Aliquots of purified total RNA served as “no reverse transcriptase” negative control to assess the amount of DNA contamination and did generally not result in a PCR product. For the analysis of RT-qPCR results, the QuantStudio software (Version 1.3; Applied Biosystems) was used. Quantitative analyses were performed according to the comparative threshold cycle method (2^DDCt method; Livak and Schmittgen 2001), using transcript levels of the β tubulin-encoding gene tubA (AFUA_1G10910) as established internal reference.

**Sequence comparison**

Identification of orthologs for all MAT1-1-1 target genes reported for *P. chrysogenum* (Becker et al. 2015) in *A. fumigatus* was performed by a reciprocal BLASTP analysis of the corresponding amino acid sequences using the publicly available genome annotations of *P. chrysogenum* Wisconsin 54-1255 (van den Berg et al. 2008), *A. fumigatus* A1163 (Fedorova et al. 2008), and *A. fumigatus* A293 (Nierman et al. 2005), as described previously (Autschl 1997; Traeger et al. 2013). The orthologs of *P. chrysogenum* MAT1-1-1 targets in *A. fumigatus* were compared to the recently
published list of 59 upregulated genes in MAT1-1-1 overexpression strain MAT1-1 OE in the A. fumigatus D141 background (Yu et al. 2018). Selected matching hits were used for sequence motif analyses.

**In silico sequence motif analysis**

Sequence motif analyses were performed by using the programs MEME (Bailey and Elkan 1995) and FIMO (Grant et al. 2011), both parts of the MEME Suite (Bailey et al. 2009). Therefore, the 1000 nt regions upstream of all A. fumigatus A1163 coding sequences were determined by custom made Perl scripts and were considered as putative promoter sequences. Identification of putative A. fumigatus MAT1-1-1 (AfMAT1-1-1) binding sites within the promoter sequences was performed using the previously reported P. chrysogenum MAT1-1 (PcMAT1-1-1) binding motif MAT1.1 (Becker et al. 2015) as the input for FIMO (p-value < 0.0001). For a *de novo* motif search, we used MEME with the zoops (zero or one de novo) parameter.

**Oligonucleotides used in electrophoretic mobility shift assays**

In total, 1000 nt upstream regions of the genes ppaA, preA, and tomA were used to design 29-bp long oligonucleotide DNA probes for electrophoretic mobility shift assays (EMSAs). The annealing of oligonucleotides was performed by combining equimolar amounts of complementary DNA as described in Becker et al. (2015). After annealing, double-stranded (ds) oligonucleotides were labeled using [γ-32P] ATP (6000 Ci/mmol; Hartmann Analytic, Braunschweig, Germany) and T4 polynucleotide kinase (Promega, Madison, WI, USA). Oligonucleotides (Supplementary Table S1) were purchased by Eurofins Genomics (Ebersberg, Germany).

**EMSAs and shift-western analysis**

EMSAs and shift-western analyses were performed as described by Becker et al. (2015) with the following minor exceptions. 32P-labeled dsDNA oligonucleotides (~100 cps) were incubated with varying protein concentrations in the presence of 8 μl binding buffer [125 mM phosphate buffer, 375 mM KCl, 0.25% bovine serum albumin (w/v), 0.25% Tween 20 (w/v), 12.5% glycerol, 12.5 ng poly (dl/dC)] (Thermo Scientific, Waltham, MA, USA) in a final volume of 20 μl at room temperature for 20 min. The same absolute amounts of the samples (20 μl) were resolved on 15% polyacrylamide gels at 4°C. The GST-AfMAT1-1-1-178-235 binding efficiency was determined in mutational analysis by measuring the signal intensities using ImageJ (Schneider et al. 2012). The DNA-binding affinity of GST-AfMAT1-1-1-178-235 was determined using the relation: Fraction bound/Fraction bound + unbound. Protein complexes with the mutated DNA samples were normalized to the complexes formed between protein and non-mutated DNA samples. Quantification of EMSA gels to determine the DNA-binding affinity of GST-AfMAT1-1-1-178-235 and AfMAT1-1-1-78-235 was carried out using ImageJ (Schneider et al. 2012).

**Data availability**

Analyzed data are provided in the attached supplementary files. Plasmids and sequences are available upon request.

Supplementary material is available at figshare DOI: https://doi.org/10.25387/g3.13122746.

**Results**

**Bioinformatic analyses identify putative AfMAT1-1-1 binding sites within the upstream regulatory regions of target genes**

Based on the *A. fumigatus* Af293 genome annotation (Nierman et al. 2005), 1000 nt regions upstream of translation initiation sequences of each *A. fumigatus* gene were used to find individual motifs of the *P. chrysogenum* MAT1-1-1 (PcMAT1-1-1) binding motif (MAT1.1) using FIMO, part of the MEME suite 5.1.1 (Bailey et al. 2009). We identified MAT1.1 in 1731 (14.2%) of 9630 upstream regions of *A. fumigatus* Af293. We then narrowed our search by selecting sex and development related genes from three different data sets: 59 differentially overexpressed genes found in a transcriptome study from an *A. fumigatus* MAT1-1 overexpression strain (Yu et al. 2018), 81 genes known to be involved in sexual reproduction in other aspergilli (Dyer and O’Gorman 2012), and 243 genes previously identified to be targets of the *P. chrysogenum* MAT1-1-1 transcription factor (Becker et al. 2015). Using the intersection of the 1731 putative target genes and genes that were found in at least two of the three afore mentioned data sets, we...
identified the 18 genes that were viewed as the most promising candidates for AfMAT1-1-1 binding (Table 1).

Among the genes identified, we found sexual development related genes, such as ppgA (AFUB_072280) and preA (AFUB_055410), encoding a sex pheromone and a pheromone receptor, and cp9 (AFUB_064360), encoding a putative serine carboxypeptidase. The fourth gene, tomA (AFUB_063230) encodes a putative protein of unknown function, which has homologs in different members of the Eurotioomycetes (Supplementary Figure S1).

Other potential targets of AfMAT1-1-1 required for functional sexual development include DmtA, which encodes DNA methyltransferase A involved in fruiting body development (Lee et al. 2008); SiaA and FhbA, encoding a phosducin-like protein required for G-protein signaling (Seo et al. 2008); and Ste7, a MAP kinase kinase that acts as an activator of pheromone transduction pathway (Bayram et al. 2012), and the transcription factor FhpA required for cleistothecia production (Dyer and O’Gorman 2012) (Table 1).

To validate the mating-type locus-controlled transcriptional expression of selected candidates, transcript levels of ppgA, tomA, cp9, and AFUB_070880 were quantified in MAT1-1 wild-type and mat1-1A deletion strains, as described in the Materials and methods section. RT-qPCR data confirmed the AfMAT1-1-1-dependent expression of these genes (Figure 1A). Based on this, and since the upstream regions of ppgA, tomA, and preA showed at least one putative binding site match with the AfMAT1-1-1 binding motif (Figure 1B), we analyzed the interaction between these exemplary promoters and the AfMAT1-1-1 transcription factor in more detail.

### Cloning and expression of variants of the AfMAT1-1-1 protein

To determine whether AfMAT1-1-1 binds to the discovered binding sites in the upstream regulatory regions of ppgA, preA, and tomA, we set out to generate soluble AfMAT1-1-1 recombinant protein for in vitro DNA-binding assays. The full-length AfMAT1-1-1 cDNA was fused with a glutathione S-transferase (GST-tag) at the N-terminus (Supplementary Figure S2). The two protein moieties were linked by a 1.8 kDa polylinker carrying a tobacco etch virus (TEV) protease recognition site (Figure 2A). Although the GST-AfMAT1-1-1 protein with a predicted molecular weight of 68.6 kDa was expressed, we found that the recombinant protein was predominantly in the insoluble fraction, and western blot analysis indicated that its expression level was low (Figure 2B).

We used several approaches to improve expression rate and solubility of full-length AfMAT1-1-1. At first, we examined the AfMAT1-1-1 sequence for the presence of codons that are rarely used in E. coli. Analysis of the native AfMAT1-1-1 gene revealed eight rarely used codons in E. coli (Supplementary Figure S3). The corresponding triplets encode for amino acids arginine, proline, leucine and the “UGA” stop codon. Therefore, we expressed the full-length AfMAT1-1-1 and C-terminally shortened variant GST-AfMAT1-1-1 encoded in plasmid pROSETTA2 (Supplementary Figure S3). The corresponding triplets encode for amino acids arginine, proline, leucine and the “UGA” stop codon. Therefore, we expressed the full-length AfMAT1-1-1 and C-terminally shortened variant GST-AfMAT1-1-1 in E. coli strain Rosetta, which carries a plasmid carrying a tobacco etch virus (TEV) protease recognition site (Figure 2A). Although the GST-AfMAT1-1-1-1 protein with a predicted molecular weight of 68.6 kDa was expressed, we found that the recombinant protein was predominantly in the insoluble fraction, and western blot analysis indicated that its expression level was low (Figure 2B).

To avoid constructing non-functional truncated proteins, the alignment of amino acid sequences in the extended alpha-box domains was revised using Jpred (Drozdzekiy et al. 2015) and IUPred2A (Mézás et al. 2018) servers (Supplementary Figure S5). Secondary structure prediction of AfMAT1-1-1 suggested

### Table 1 MAT1-1 motif occurrence in the promoter regions of genes regulated by AFMAT1-1-1 or other sexually associated genes from Aspergillus species

| ID          | Description          | # of motifs | Distance to TIS | DNA strand | Sequence (5’→3’) | Motif p-value | ΔMAT1-1-1 DE (qRT-PCR, 2^−ΔΔCt) | ΔMAT1-1-1 OE (RNA-seq, log2) |
|-------------|----------------------|-------------|----------------|------------|-----------------|---------------|---------------------------------|------------------------------|
| AFUB_072280 | mating alpha-pheromone PpgA | 1           | −315           | +          | TTATTGAG        | 6.14E-05      | 2003.3                          | 8.11                         |
| AFUB_063230 | conserved hypothetical protein TomA | 2           | −380, −406     | −          | TTATTGAT        | 3.96E-05      | 268.8                           | 7.97                         |
| AFUB_064360 | serine carboxypeptidase, putative C99 | 2           | −238, −254     | +          | TTATTGAT        | 1.79E-05      | 255.1                           | 5.67                         |
| AFUB_055410 | a-pheromone receptor PreA         | 2           | −244, −519     | −          | TTATTGAT        | 1.79E-05      | nd                             | 5.41                         |
| AFUB_070880 | conserved hypothetical protein     | 2           | −248, −457     | +          | TTATTGAT        | 3.96E-05      | 286.4                           | 5.13                         |
| AFUB_016640 | comidal conophobin RodB           | 1           | −392           | −          | TTATTGAT        | 6.14E-05      | nd                             | 4.35                         |
| AFUB_094560 | DNA methyltransferase-like DmtA   | 1           | −867           | +          | TTATTGAT        | 6.14E-05      | nd                             | 1.91                         |
| AFUB_032200 | protein kinase, putative          | 1           | −103           | +          | CTATTGAT        | 7.93E-05      | nd                             | 1.76                         |
| AFUB_021310 | maleylacetacetate isomerase MapiA | 1           | −593           | +          | CTATTGAT        | 1.79E-05      | nd                             | 1.74                         |
| AFUB_007030 | conserved hypothetical protein     | 2           | −609           | +          | CTATTGAT        | 3.96E-05      | nd                             | 1.69                         |
| AFUB_078190 | conserved hypothetical protein     | 1           | −870           | −          | CTATTGAT        | 3.96E-05      | nd                             | 1.64                         |
| AFUB_021320 | transcription factor (zinc-finger) | 1           | −687           | +          | CTATTGAT        | 1.79E-05      | nd                             | 1.61                         |
| AFUB_070440 | oxidoreductase, putative           | 1           | −42             | −          | CTATTGAT        | 1.79E-05      | nd                             | 1.60                         |
| AFUB_095808 | ferric-chelate reductase, putative | 2           | −328, −436     | −          | CTATTGAT        | 7.93E-05      | nd                             | 1.59                         |
| AFUB_099650 | NO response (flavohaemoglobin) FhbA | 1           | −878           | +          | CAATTGAT        | 9.72E-05      | nd                             | −1.0                         |
| AFUB_043130 | MAP kinase, kinase STE7           | 1           | −649           | +          | CTATTGAT        | 3.96E-05      | nd                             | 0.24                         |
| AFUB_020110 | transcription factor (forkhead) FhpA | 1           | −732           | +          | CTATTGAT        | 3.96E-05      | nd                             | −1.08                        |
| AFUB_059790 | phosducin-like protein PhmA         | 1           | −454           | −          | CAATTGAT        | 9.72E-05      | nd                             | −0.1                         |

*As obtained from the AspGD website: http://www.aspergillusgenome.org/.

TIS, translational initiation site.

Aspergillus nidulans orthologs, associated with sexual reproduction in A. nidulans (Dyer and O’Gorman 2012).

n-Fold transcript levels in Aspergillus fumigatus MAT1-1-1 wild-type isolate in comparison to its congenic mat1-1-1A deletion strain.
that the conserved alpha-box domain contains three helices. A fourth helix is predicted adjacent to the alpha-box (Supplementary Figure S5A). The alpha-box domain (amino acids 78–139) together with the downstream sequence extending to amino acid 184 form a so-called MATalpha_HMG-box (Pfam PF04769) domain, which was defined previously (Martin et al. 2010). The central region of AfMAT1-1-1 (amino acids 83–218), including the MATalpha_HMG-box domain, is predicted to be globular, whereas the N- and C-terminal parts of the protein appear to be naturally disordered (Supplementary Figure S5B).

Considering the secondary structure and globularity of AfMAT1-1-1, we designed a set of protein constructs with truncated N- and/or C-termini, which all contained the complete MATalpha_HMG-box domain (Figure 2A). We tested the variant constructs for optimal protein synthesis and binding activity toward tom1-2 and tomA-1 probes (Supplementary Figure S6). The DNA-binding activity of the variant AfMAT1-1–235, expressing the complete N-terminal part with DNA-binding domain was not significantly altered in comparison with variants AfMAT1-1–218 and AfMAT1-1–178 encoding only the region of

![Figure 1](image-url)
DNA-binding domain. Finally, we have chosen construct GST-AfMAT1-1-178–235 for a detailed biochemical analysis, since this is the smallest peptide and showed the best solubility and minimal protein degradation after purification from E. coli (Figure 2C).

AfMAT1-1-178–235 remains stable in solution after removal of the GST-tag

AfMAT1-1-178–235 contains the entire MATalpha_HMG-box domain with an additional 51 residues at the C-terminus (Figure 2A). Following high-yield purification (see Supplementary File S2), we noted that after SDS-PAGE analysis the final GST-AfMAT1-1-178–235 product appeared approximately 7 kDa smaller than the predicted 44.1 kDa (Figure 3A and B). We subsequently removed the GST-tag and after a second purification, GST-tag and GST-tagged TEV were separated from AfMAT1-1-178–235 (Figure 3C). After size-exclusion chromatography (Figure 3D), AfMAT1-1-178–235 eluted from a Superdex™ 75 size-exclusion column as a rather symmetric peak corresponding to a molecular weight of about 28.4 kDa (Supplementary Figure S7). Since the theoretical molecular weight of AfMAT1-1-178–235 was calculated to be 18 kDa, the protein is likely to exist as a dimer in aqueous solution. SDS-PAGE analysis of eluted fractions indicated that protein migrates as a single band, confirming that the purified protein was nearly homogeneous (Figure 3D). Our yields of pure protein estimated from a Bradford protein assay were on average 30–35 mg per 1 l of bacterial culture.

In vitro binding specificity of GST-AfMAT1-1-178–235

We examined sequence-specific DNA binding of the GST-AfMAT1-1-178–235 using a radiolabeled 29-bp dsDNA probes in EMSA. The oligonucleotide sequences were based on the ppgA, preA, and tomA promoters and they all contained the predicted MAT1.1 motif (Figure 1C and Supplementary Table S1). GST-AfMAT1-1-178–235 bound to all the dsDNA oligonucleotides (Figure 4, A–C), including the dsDNA oligonucleotides ppg1-2 and tom1-2, originating from the P. chrysogenum promoters of ppg1 and tom1 genes (Figure 4, A and C). The ppg1-2 and tom1-2 were shown previously to be bound by PcMAT1-1-1 (Becker et al. 2015). In contrast, the dsDNA oligonucleotides derived from the A. fumigatus ppgA and tomA promoters were not bound by the PcMAT1-1-1 ortholog (Figure 4, A and C). Taken together, this analysis indicates that the protein region from 78 to 235 amino acids encoded by AfMAT1-1-1-178–235 is sufficient for forming DNA-protein complexes. Based on the dsDNA probes bound by GST-AfMAT1-1-178–235, we redefined the consensus binding motif as (T/O/T/C)ATTGA(G/T/C) (Figure 4D). We conclude that the AfMAT1-1-178–235 peptide tolerates variation in the nucleotides flanking the core “ATTGA” of the binding motif.

To address the question of whether AfMAT1-1-178–235 acts in a sequence-specific manner, we introduced nucleotide substitutions within the AfMAT1-1-1-178–235 binding motif in dsDNA oligonucleotides ppgA-1 and tomA-1 (Figure 5A). Nucleotide A in position 3 of the consensus binding motif “TTATTGAG” (ppgA-1) and “CTATTGAT” (tomA-1) was substituted by a G, resulting dsDNA oligonucleotide probes ppgA_1_m1 and tomA-1_m1, and then further substitution of nucleotide G in position 6 to an A resulted in oligonucleotides ppgA-1_m2 and tomA-1_m2 (Supplementary Table S1). The binding efficiency of GST-AfMAT1-1-178–235 was quantified by EMSAs (Figure 5, A and B). The first single nucleotide substitution at position 3 in the motif significantly reduced binding of GST-AfMAT1-1-178–235. Binding decreased by 50% for tomA-1_m1 and 90% for ppgA-1_m1 compared to the wild-type sequences. Unexpectedly, the second nucleotide substitution in oligonucleotides ppgA-1_m2 and tomA-1_m2 did not further reduce binding of GST-AfMAT1-1-178–235. Thus a single nucleotide change within the AfMAT1-1 binding motif at position 3 significantly alters binding of GST-AfMAT1-1-178–235, indicating that binding of AfMAT1-1-178–235 to DNA is sequence specific (Figure 5B).

To confirm the observations above, we tested the DNA sequence specificity of the protein domain by performing a competition assay (Figure 5C). Non-radiolabeled ppgA-1 served as a specific competitor in EMSAs, preventing non-specific nucleotide-protein interactions, while non-radiolabeled dsDNA oligonucleotide ppgA-1_m2 was used to investigate the effect of mutations in the AfMAT1-1 binding site on protein binding. Incubating GST-AfMAT1-1-178–235 with increasing concentrations of specific competitor oligonucleotide ppgA-1 significantly decreased the signal intensity of the shifted bands (Figure 5C, left
However, increasing concentrations of the non-specific competitor ppgA-1_m2 only slightly decreased the signal intensity, thus confirming our results with the specific competitor. Western analysis using an anti-GST antibody further confirmed that oligonucleotide DNA binding is mediated by the GST-AfMAT1-1-178–235 protein (Figure 5C, right panel). In summary, our results show that binding of GST-AfMAT1-1-178–235 to ppgA-1 and tomA-1 target motifs is sequence specific. Additionally, the third A nucleotide of the consensus motif \((T/C)\)TATTGA(G/T) seems to be important for AfMAT1-1-178–235 sequence-specific DNA binding.

Next, we assessed the binding affinity of GST-AfMAT1-1-178–235 and GST-free AfMAT1-1-178–235 by determining the equilibrium dissociation constants (K_D). Increasing amounts of purified proteins were incubated with the fixed concentration of dsDNA oligonucleotide tomA-1 (Figure 6). Both proteins interacted with the target DNA, which decreased the amount of radiolabeled tomA-1 probe (Figure 6, A and B). The binding affinity of the proteins was...
quantified using an established method (Heffler et al. 2012) and was further evaluated by nonlinear regression analysis. The $K_D$ values of 1.45 ± 0.26 and 1.83 ± 0.1 μM were obtained for GST-AfMAT1-1-178–235 and AfMAT1-1-178–235. Since the $K_D$ values of the proteins were very similar, DNA binding of AfMAT1-1-178–235 was not significantly altered by removal of the GST-tag.

**Discussion**

Here, we used a bioinformatics analysis, together with biochemical and DNA-binding studies to answer the question of whether the AfMAT1-1-1 binding sites can be defined based on the known binding motif of the MAT1-1-1 protein from *P. chrysogenum*, a closely related member of Eurotiales. After considering the codon usage of the recombinant fungal gene and choosing different *E. coli* host strains, we found that a N-terminally tagged AfMAT1-1-1-178–235 construct, containing the MAT alpha-box domain, was best suited for our in vitro DNA-binding assays. The purified AfMAT1-1-1 construct interacts specifically with the promoter sequences from *ppgA* and *tomA*, which were shown to be strongly transcriptionally regulated in a *A. fumigatus* overexpression strain (Yu et al. 2018). From our result we conclude that the...
N-terminal part of AfMAT1-1-1 has no direct impact on DNA-binding specificity. However, we cannot exclude, that the C-terminal part of AfMAT1-1-1 increases the DNA-binding specificity, since we were unable to express variant AfMAT1-1-178–368, which encodes the complete C-terminal part of the protein.

Sexual development in ascomycetes is controlled by mating-type locus encoded transcription factors that carry either a HMG or an alpha-box domain. While HMG domains are ubiquitous and their protein–DNA interactions are molecularly well defined (Philley and Staben 1994; Štros et al. 2007; Ait Benkhali et al. 2013), alpha-box domain transcription factors have a rather limited distribution and are less well studied. The alpha-box domain orthologs from ascomycetes are very similar; however comparative studies of alpha-box domain proteins from the two protoascomycetes Saccharomyces cerevisiae and Candida albicans showed that the DNA-binding specificity of the C. albicans Matα1 changed after the divergence of S. cerevisiae and C. albicans (Carr 2004; Tuch et al. 2008; Baker et al. 2011). In S. cerevisiae, the transcription of

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**Figure 5** Single nucleotide substitution in the core binding motif decreases DNA binding of GST-AfMAT1-1-1-178–235 protein. (A) EMSA of the GST-AfMAT1-1-1-178–235 incubated with different radiolabeled dsDNA probes, as indicated. (B) Comparison of GST-AfMAT1-1-1-178–235 DNA-binding activity binding between the non-mutated and mutated DNA probes. Shown is the relative intensity determined from the autoradiograms as described in the Materials and methods section. Graphs show mean ± SD (n = 3). The p-values are indicated. Student’s t-test. (C) Competition experiment, using radiolabeled ppgA-1 in the presence of unlabeled ppgA-1 (specific competitor) and unlabeled ppgA-1_m2 (non-specific competitor). Unlabeled probes with increasing concentration from 2.5 to 16.5 μM were incubated with the protein. Shift-Western experiment (GST-immunoblot) demonstrated the presence of GST-AfMAT1-1-1-178–235/DNA complexes. In total, 4 μg (± 4.5 μM) of protein was used per each reaction. Arrow heads indicate formation of protein–DNA complexes.
Specific genes required for mating-type specification is activated by the alpha-box domain transcription factor MATα1. However, the transcriptional activation of the MATα1 target genes in S. cerevisiae and C. albicans is only achieved when the MADS-box transcription factor Mcm1 forms a complex with MATα1 (Carr 2004). This probably explains a faster divergence of alpha1 DNA-binding specificity in these two yeasts compared to the two euascomycete compared in this study. In P. chrysogenum, the full-length alpha-box domain transcription factor MAT1-1-1 itself is sufficient for sequence-specific DNA binding (Becker et al. 2015). The fact that A. fumigatus and P. chrysogenum are phylogenetically closely related and that MAT1-1-1 orthologs from these two species share some common target genes explains why their binding motifs are at least partially conserved (Becker et al. 2015; Yu et al. 2018).

We succeeded in purifying a truncated version of AfMAT1-1-1, where the C-terminal 133 amino acids residues were deleted. This modification improved significantly the solubility of the construct compared to the full-length AfMAT1-1-1 protein. We speculate that the presence of a long intrinsically disordered region in the C-terminus (amino acids 268–294) of AfMAT1-1-1 could cause low solubility of the protein in aqueous solutions (Supplementary Figure S3B). This notion is consistent with studies where long intrinsically disordered regions were shown to be abundant in eukaryotic transcription factors and proved to be prone to aggregation (Ward et al. 2004; Uemura et al. 2018).

Using the most soluble protein construct, we show that the extended alpha-box domain of AfMAT1-1-1 interacts specifically with the promoter sequences from ppgA, preA, and tomA in vitro. Thus, confirming that the main components of the pheromone response pathway ppgA and preA are direct targets of the A. fumigatus MAT1-1-1 protein. This is consistent with data obtained for Matα1 transcription factor from S. cerevisiae (Galgoczy et al. 2004) or P. chrysogenum (Becker et al. 2015). Interestingly, we detected a conserved target of MAT1-1-1, tomA, which was previously discovered in two independent investigations (Becker et al. 2015; Yu et al. 2018). Here, tomA was one of the top targets of AfMAT1-1-1. In P. chrysogenum, the tomA ortholog (Pc20g00090) showed the highest statistically significant peak value in a PcMAT1-1-1 ChIP-seq analysis (Becker et al. 2015), while in A. fumigatus, tomA showed the highest differential expression in a comparative transcriptome analysis between a MAT1-1-1 overexpression strain and the wild type (Yu et al. 2018). TomA orthologs are generally present in members of the Eurotiomycetidae, a subclass of the ascomycetes, which besides Aspergillus and Penicillium species also includes the human pathogens Histoplasma capsulatum, Coccidioides spp, and Blastomyces dermatitidis. The conserved putative NLS “RKRRA” was identified at the N-terminus of TomA orthologs, suggesting TomA is a nuclear protein (Supplementary Figure S1). Other genomic targets, such as conidial hydrophobin RodB, DNA methyltransferase-like DmtA, and transcription factor SilA, also

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**Figure 6** Determination of the equilibrium dissociation constants for GST-AfMAT1-1-178–235 and AfMAT1-1-178–235. (A, B left panels) EMSA titrations of the purified proteins with the tomA-1. Increasing concentrations of proteins GST-AfMAT1-1-178–235 (1–7.5 μM) and AfMAT1-1-178–235 (1–6.5 μM) were incubated with the radiolabeled tomA-1 dsDNA oligonucleotide. Protein/DNA complexes were separated through a native polyacrylamide gel, and the radiation signals from each band were obtained from the EMSA profiles. The intensity of the signals was quantified using ImageJ (Schneider et al. 2012). Arrow heads indicate the position of the protein–DNA complexes. (A, B right panels) Determination of the equilibrium dissociation constant (K\(_D\)) for the GST-AfMAT1-1-178–235 and AfMAT1-1-178–235. The fractions of bound and free DNA from A (for GST-AfMAT1-1-178–235) and B (for AfMAT1-1-178–235) were plotted as a function of the protein concentration and fitted with nonlinear regression analysis. The averaged K\(_D\) values from three assays with the standard error for each protein, GST-AfMAT1-1-178–235 and AfMAT1-1-178–235, are shown in the plot.
appear to be direct targets of AfMAT1-1-1, which is in line with findings that MAT1-1-1 proteins have highly diverse functions in developmental processes. Future ChIP-seq experiments will be necessary to identify all target genes of MAT1-1-1 transcription factors in vivo.

MAT1-1-1 transcription factors from A. fumigatus and P. chrysogenum share reasonable similarity, with 36% sequence identity, whereas the alpha-boxes themselves have 58% amino acids in common (Supplementary Figure S5). Thus, the alpha-box domains are the most conserved region within MAT1-1-1 orthologs from diverse ascomycetes (Martin et al. 2010), yet any conservation of regions extending outside these domains is extremely limited. A previous study demonstrated that the transcription factor DNA-binding preference can be predicted on the basis of amino acid sequence similarity within other closely related members of the same transcription factor family (Alleyne et al. 2009). For example, cross-species conservation of regulatory regions of transcription factors was shown for human and mouse HMG domains from Sox proteins (Kamachi and Kondoh 2013). Moreover, preservation of cis-regulatory elements in orthologous promoter regions was observed in closely related ascomycetes (Casch et al. 2004). The binding motifs of MAT1-1-1 proteins from A. fumigatus and P. chrysogenum are highly similar. The core “A3T4T5G6A” sequence is present in both; however, variations of nucleotides occur outside the central consensus motif. Furthermore, the same core sequence is also recognized by the MATα1 in S. cerevisiae (Baker et al. 2011). A single base pair substitution of the first A3 to a G within the AfMAT1-1-1 core consensus motif in ppgA-1 and tomA-1 oligonucleotides led to significantly decreased DNA binding, indicating the importance of this nucleotide for the DNA-binding activity of the protein. Surprisingly, an additional nucleotide substitution (G6 &cenve_unknown_entity_wingdings3_F022; A) did not further alter the binding of AfMAT1-1-1-178–235. Similarly, a single nucleotide substitution of the third base within the core of MAT1-1-1 binding motif severely diminished formation of the protein–DNA complexes in P. chrysogenum (Becker et al. 2015). Therefore, we reasoned that nucleotides present in the core motif are important for the binding of MAT1-1-1 orthologs.

In summary, our study revealed that the DNA-binding motif of MAT1-1-1 from A. fumigatus resembles the motif reported for MAT1-1-1 from P. chrysogenum. Using a purified transcription factor fragment, we gained new insights into the molecular basis of DNA binding by fungal MAT alpha-box domains. The identification of putative AfMAT1-1-1 binding sites within the upstream regulatory regions of conserved target genes strengthens the idea that this process is highly conserved within members of Eurotiales (Eurotiomycetidae), despite their multiple lifestyle transitions. Additional in vivo studies will prove whether shortened versions of the MAT1-1-1 locus are sufficient to restore fertility in the sterile AMAT1-1-1 strain of A. fumigatus.

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