A Network of HMG-box Transcription Factors Regulates Sexual Cycle in the Fungus *Podospora anserina*

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**Abstract**

High-mobility group (HMG) B proteins are eukaryotic DNA-binding proteins characterized by the HMG-box functional motif. These transcription factors play a pivotal role in global genomic functions and in the control of genes involved in specific developmental or metabolic pathways. The filamentous ascomycete *Podospora anserina* contains 12 HMG-box genes. Of these, four have been previously characterized; three are mating-type genes that control fertilization and development of the fruit-body, whereas the last one encodes a factor involved in mitochondrial DNA stability. Systematic deletion analysis of the eight remaining uncharacterized HMG-box genes indicated that none were essential for viability, but that seven were involved in the sexual cycle. Two HMG-box genes display striking features. *PaHMG5*, an ortholog of *SpSte11* from *Schizosaccharomyces pombe*, is a pivotal activator of mating-type genes in *P. anserina*, whereas *PaHMG2* is a repressor of several phenomena specific to the stationary phase, most notably hyphal anastomoses. Transcriptional analyses of HMG-box genes in *Podospora anserina* showed that *PaHMG5* and its upstream regulator *SRY* are involved in sexual reproduction in fungi, as 11 out of 12 members were involved in the sexual cycle in *P. anserina*. *PaHMG5* and *SpSte11* are conserved transcriptional regulators of mating-type genes, although *P. anserina* and *S. pombe* diverged 550 million years ago. Two HMG-box genes, *SOX9* and its upstream regulator *SRY*, also play an important role in sex determination in mammals. The *P. anserina* and *S. pombe* mating-type genes and their upstream regulatory factors form a module of HMG-box genes analogous to the SRY/SOX9 module, revealing a commonality of sex regulation in animals and fungi.

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**Introduction**

High-mobility-group box (HMGB) proteins [1] include chromatin architectural proteins as well as specific transcription factors that are involved in highly diverse functions ranging from sex determination [2] to extracellular immune signaling [3,4]. All of these functions rely on the HMG-box, a conserved motif containing approximately 80 amino acids arranged in a distinctive L-shaped three-α-helical fold [5,6]. This HMG-box motif sharply bends DNA [7] and facilitates the assembly of transcriptional complexes that involve other proteins by distorting chromatin [8]. Based on phylogenetic analyses of the HMG-box, the HMGB superfamily can be divided into two families, HMGB-UBF_HMG and SOX/TCF/MATA_HMG [9]. These families were named after the best known representative in each group, namely hUBF [10], the SOX (Sry-type HMG-box) genes [11], TCF-1 [12] and MATa-1 [13]. The HMGB-UBF_HMG family is considered to be sequence non-specific, as generic UBF proteins can bind both ribosomal DNA regulatory sequences and sequences across the entire ribosomal DNA repeat [14]. Members of this family are present in plants, fungi and animals. On the other hand, the SOX/TCF/MATA_HMG family contains proteins that bind specific DNA sequences, with a common T/A rich core (reviewed in [2]). The SOX/TCF/MATA_HMG family is subdivided into the SOX/TCF_HMG and the MATA_HMG subfamilies. The MATA_HMG subfamily includes exclusively fungal proteins, most of which are involved in sexual processes, while SOX-TCF_HMG genes appear to be restricted to animals [9].

Members of the HMGB-UBF_HMG family function in many processes such as transcription, genomic stability and the three R’s (replication, recombination and DNA repair) (reviewed in [8]). An exhaustive analysis of HMGB-UBF_HMG-box encoding genes was performed in *Saccharomyces cerevisiae* in a previous study, and it showed HMGB-UBF_HMG-box genes to have highly diversified functions. In brief, the six HMGB-UBF_HMG-box genes from *S. cerevisiae* are involved in ribosomal DNA transcription [15],
chromatin remodeling complexes [16,17], and mitochondrial metabolism and energy [18,19,20]. Analysis of HMG-UBF/HMG-box genes in *S. pombe* mainly focused on the *splsd1* and *splsd2* genes. The SplLd1 protein functions as a histone demethylase, and both SplLd1/2 are believed to affect the epigenetic state of the cell [21].

Members of the SOX/TCF/MATA_HMG family are important regulators of differentiation and the sexual process. In mammals, the Y-linked testis-determining factor, SRY, is the founding member of the SOX subfamily [11]. SRY induces male sex determination by regulating *Sox9* [22,23,24]. Unlike *Syr*, *Sox9* is conserved among non-mammalian vertebrates and induces male-to-female sex-reversal when mutated [25], illustrating that it is ancestral and critical for vertebrate function. HMG-box genes also play a central role in the fungal mating process. Almost all mating-type loci from Ascomycota contain at least one MATA_HMG gene [26,27]. The most notable exception to this is *S. cerevisiae* (Saccharomycotina), which lost one MATA_HMG mating-type gene during evolution [28] but retained the prototypical α1 protein MATα1p. MATα1p was identified as a member of the MATA_HMG subfamily [27]. *ROX* is the other gene of this subfamily in *S. cerevisiae* and one of the few fungal MATA_HMG-box genes that are not involved in sexual reproduction. Instead, it represses the expression of hypoxic genes [29]. In *S. pombe*, mating-type gene transcription is regulated by the MATA_HMG-box gene, *SpSte11* [30]. The activation of the sexual process in *S. pombe* relies on the nuclear accumulation of SpSte11, which is triggered by starvation and pheromone signaling [31]. An allelic system of mating-type genes functions in the yeast *Saccharomyces* but are not involved in sexual reproduction. Instead, it plays an essential role in sexual development, as shown in *Ustilago maydis* [34,36], but they were part of the SOX-TCF_HMG and HMG-UBF/HMG-box groups [27]. In Basidiomycota, mating-type loci do not contain HMG-box genes, but *MATa_HMG-box* genes play an essential role in sexual development, as shown in *Ustilago maydis* [37,38,39], *Catenopsis cinerea* [40] and *Cryptococcus neoformans* [41,42].

A genome-wide systematic deletion analysis of transcription factors performed in the homothallic fungus *Podospora anserina* indicated that several HMG-box genes are involved in sexual development [43]. However, not all HMG-box genes from *F. graminearum* were deleted in that study and their genetic relationships were not investigated. Here, we report the systematic deletion analysis of HMG-box genes in the heterothallic fungus *Podospora anserina* as well as an in-depth analysis of their genetic interactions. The *P. anserina* genome encodes a total of 12 HMG-box genes. Of these, four have been previously characterized, including three mating-type genes (*FPR1, EMR1* and *SMR2*) belonging to the MATA_HMG subfamily [27], and the nuclear gene encoding a mitochondrion-targeted HMG-UBF-box protein (*mtHMG1, Pa_1_13340*) [44] (Table 1). *FPR1* is encoded by the *mai+* idiomorph [45], while *EMR1* and *SMR2* are both encoded by the *mai–* idiomorph [45,46]. These mating-type genes control fertilization and the development of the fruit-body (reviewed in [47]). The remaining eight uncharacterized HMG-box genes encode two MATA_HMG-box proteins, four HMG-UBF/HMG-box proteins, and two proteins that contain an HMG-box with an atypical residue. In the present study, these genes were individually deleted and the phenotypes of mutant strains were carefully assessed. The results revealed that all genes were dispensable for viability. Moreover, six mutant strains displayed sexual reproduction defects, and one of the two remaining mutants displayed vegetative defects. Transcriptional analyses indicated that an ortholog of *SpSte11* is at the hub of the HMG-box gene network and that it regulates the expression of *EMR1* and *FPR1* and fertility.

**Results**

**Identification of HMG-box genes in *P. anserina***

The *Podospora anserina* genome database (Materials and Methods) was searched for HMG-box proteins using the previously defined HMG-box consensus sequence [27] as query in a Blastp search [48]. *P. anserina* genome annotation in Fungal Transcription Factor [49] and Superfamily databases [50] was examined to identify possible missing HMG-box genes in our analyses. A total of 12 HMG-box genes were identified. Of these, eight had not been previously characterized and were here named *PaHMG2* to *PaHMG11* (Table 1). CD-Search [51] identified five MATA_HMG-box proteins and five HMG-UBF/HMG-box proteins (Table 1), but failed to categorize two HMG-box proteins. These two proteins, *PaHMG7* and *PaHMG9*, contained an atypical charged residue instead of a conserved aliphatic or aromatic amino acid at position 9 (Figure 2). However, they contained the specific aromatic amino acids that anchor the recognition helix of the HMG-box domain to the hydrophobic core at position 8 and 11, confirming that they belong to the HMG superfamily. Additional domains are shown in Table 1. Except for *mtHMG1*, which contained a mitochondrial targeting signal, the remaining 11 HMG-box proteins from *P. anserina* were predicted to localize to the nucleus.

A search for orthologs of *P. anserina* HMG-box genes with FUNGIpath [52] in selected fungal species indicated that three had an ortholog in *S. cerevisiae*, six had an ortholog in *S. pombe*, and all had an ortholog in *Neurospora crassa* (Table 1). *PaHMG6*, *PaHMG7* and *PaHMG8* had orthologs in Basidiomycota but the functions of these orthologs have not been investigated yet. *Prf1*, *Rop1* and *HMG3* from *U. maydis* [37,38], *Mat2* from *C. neoformans* [42] and *Pec1* from *C. cinerea* [40] encoded HMG-box regulators that are related to the sexual cycle in Basidiomycota. FUNGIpath did not detect any *P. anserina* orthologs for these HMG-box genes.
Most HMG-box genes localized to chromosome 1, which contains the mating-type locus [53]. Only three out of 12 HMGB genes mapped outside of chromosome 1, suggesting that the distribution of HMG-box genes may be biased to chromosome 1. However, statistical tests indicated this bias to be inconclusive (Material and Methods).

Evolutionary analysis of fungal HMG-box proteins

The HMG-box of the 12 \textit{P. anserina} HMGB proteins was extracted and grouped in a phylogram with the HMG-box motif of selected plant, animal and fungal species, including \textit{S. cerevisiae}, \textit{S. pombe}, \textit{N. crassa}, \textit{Aspergillus nidulans}, \textit{Cochliobolus heterostrophus} and \textit{U. maydis} (Figure 2). A total of 154 HMG-box domains were clustered in four groups, which overall corresponded to the previously defined HMGB groups [9]: MAT\textsubscript{a}\textsubscript{HMG} (group A, Figure 2) [27], MATA\textsubscript{HMG} (group B, Figure 2), SOX-TCF\textsubscript{HMG} (group C, Figure 2) and HMGB-UBF\textsubscript{HMG} (group D, Figure 2).

Group A (Figure 2) included exclusively the mating-type transcription factors with an \textit{a}1 domain, which was proposed to correspond to a new class of HMG-box [27,54]. Group A formed a clade related to the MATA\textsubscript{HMG} subfamily and contained FMR1 (Podan1). The other HMG-box mating-type transcription factors FPR1 (Podan2a) and SMR2 (Podan3) were placed in group B (Figure 2), which contained mostly MATA\textsubscript{HMG}-box proteins. PaHMGG (Podan2c) clustered within clade G, with SpSte11 (Schpo2c) of \textit{S. pombe} [30] and NCU09387/FMF-1 (Neucr2c) of \textit{N. crassa} [55,56]. The orthology of these three proteins, suggested by their phylogenetic position, was independently confirmed with FUNGIPATH. Moreover, the relationship of NCU09387 (Neucr2c) with SpSte11 (Schpo2c) was previously reported [57]. PaHMGG (Podan2b) was the only MATA\textsubscript{HMG}-box protein encoded by a gene outside chromosome 1. Its ortholog in \textit{S. cerevisiae} is ROX1p (Sacce2), a repressor of hypoxic genes, and one of the few MATA\textsubscript{HMG}-box proteins not related to mating processes [29]. Earlier evolutionary analysis of the oxygen-responding system in \textit{Kluyveromyces lactis} and \textit{S. cerevisiae} suggested that ROX1 was recruited specifically to control this system in \textit{S. cerevisiae} [58]. Rfg1p (Canal2), the ortholog of ROX1p (Sacce2) in \textit{Candida albicans}, controls filamentous growth and virulence [59]. Interestingly, the clade defined by PaHMGG (Podan2b), ROX1p (Sacce2) and Rfg1p (Canal2) included a MAT1-2-1 mating-type protein (Ajeca2, see ‘&’ in clade B, Figure 2) and was placed close to the MAT1-2-1 (Podan2a, Aspni2a, Neucr2a and Sorma2) and MAT1-1-3 mating-type proteins (clade E, Figure 2). The placement of the PaHMGG (Podan2b)/ROX1p (Sacce2) clade in the MATA\textsubscript{HMG} subfamily and our functional analyses of PaHMGG (Podan2b) (see below) supports the idea that the

### Table 1. HMG-box genes in \textit{P. anserina}.

| Gene number (gene name) | Gene function | HMGB family | Additional domain | Orthologs |
|-------------------------|---------------|-------------|-------------------|-----------|
| (FMR1)                  | Mating-type regulation | MATA\textsubscript{HMG} | - | - | S. cerevisiae | S. pombe | N. crassa |
| (SMR2)                  | Mating-type regulation | MATA\textsubscript{HMG} | - | - | - | - | - |
| Pa_1_20590 (FPR1)       | Mating-type regulation | MATA\textsubscript{HMG} | - | - | Mc | MAT a-1 |
| Pa_1_13340 (mHMG1)      | Mitochondrial DNA stability | HMGB\textsubscript{UBF} | DUF1898 | - | - | - | - |
| Pa_1_7390 (PaHMGG2)     | Unknown function | HMGB\textsubscript{UBF} | SAM | - | - | - | - |
| Pa_1_9380 (PaHMGG3)     | Unknown function | HMGB\textsubscript{UBF} | SWIRM, amine-oxidase | Isd1 | - | S. cerevisiae |
| Pa_1_11050 (PaHMGG4)    | Unknown function | HMGB\textsubscript{UBF} | - | - | - | - | - |
| Pa_1_13940 (PaHMGG5)    | Unknown function | MATA\textsubscript{HMG} | - | - | ste11 | - | - |
| Pa_1_14230 (PaHMGG6)    | Unknown function | HMGB\textsubscript{UBF} | - | NHP68 | NHP6 | S. cerevisiae |
| Pa_1_8400 (PaHMGG7)     | Unknown function | HMGB | Spt1-like | - | - | N. crassa |
| Pa_1_6110 (PaHMGG8)     | Unknown function | MATA\textsubscript{HMG} | - | ROX1 | - | - | - |
| Pa_1_7190 (PaHMGG9)     | Unknown function | HMGB | - | - | - | - | - |

Figure 1. Alignment of HMG domains from the 12 HMG-box proteins of \textit{P. anserina}. The alignment was performed using ClustalW2 [105] and colored according to the Clustal X color scheme provided by Jalview [106]. This color scheme is displayed in Table S1.

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Figure 2. Unrooted phylogram for the HMG-box superfamily. Clustering of core amino acid sequences using maximum-likelihood and model LG+G [107]. Color labeling: MATα_HMG (A, green), MATA_HMG (B, yellow), SOX-TCF_HMG (C, orange), HMGB-UBF_HMG (D, H, blue), MAT1-1-3 in MATA_HMG (E, white) and STE11 in MATA_HMG (G, red). Other labels: Microsporidia MAT sex locus in HMGB-UBF_HMG (F, grey), *Phycomyces blakesleeanus* (Zygomycota) sexM (Phybl8) and sexP (Phybl9) (purple) and *P. anserina* proteins (red dots). LR-ELW values greater than 70% are shown. Abbreviations: *Ailuropoda melanoleuca* (Ailme); *Ajellomyces capsulatus* (Ajeca); *Alternaria alternata* (Altal); *Alternaria brassicicola* (Altbr); *Anopheles gambiae* (Anoga); *Antonospora locustae* (Antlo); *Arabidopsis thaliana* (Arath); *Aspergillus fumigatus* (Aspfu); *Aspergillus nidulans* (Aspni); *Botryotinia fuckeliana* (Botfu); *Bipolaris sacchari* (Bipsa); *Caenorhabditis elegans* (Caeel); *Candida albicans* (Canal); *Cereus elaphus yarkandensis* (Cerel); *Ciona savignyi* (Ciosa); *Cochliobolus heterostrophus* (Coche); *Cochliobolus homomorphus* (Cocho); *Coprinopsis cinerea* (Copci); *Cryptophyllum parasitica* (Crypa);
ancestral ROXI gene was primarily involved in sexual development before being recruited for various other functions in \textit{S. cerevisiae} and \textit{C. albicans}. Surprisingly, group B contained an HMG-UBF_HMG clade \textit{H. yabusa} (\textit{Homo sapiens}) that is conserved in \textit{NCU07568} (\textit{Neucr6a}) and several fungal orthologs, notably the histone demethylase \textit{SpLsd1} (\textit{S. pombe}) [21]. All proteins from clade \textit{H} have SWIRM and amino oxydase domains, which are characteristic of histone demethylases, thus supporting their orthologous relationship.

Five \textit{P. anserina} HMG-box proteins were classified into group D (Figure 2), which comprised members of the HMG-UBF_HMG family, except the clade members \textit{PaHMG7} (\textit{Podan6b}), \textit{NCU06874} (\textit{Neucr6b}) from \textit{N. crassa} and \textit{NP_595970} (\textit{Schpo7b}) from \textit{S. pombe}. These three proteins are characterized by a conserved atypical residue in the HMG-box domain [see the above section for its description in \textit{PaHMG7} (\textit{Podan6b}) and a SprT-like domain (Interpro accession number IPR006640).

The four HMG-UBF_HMG-box proteins, \textit{PaHMG2} (\textit{Podan5c}), \textit{PaHMG4} (\textit{Podan5b}), \textit{mthMG1} (\textit{Podan5d}), and \textit{PaHMG6} (\textit{Podan5a}), branched into different clades of group D. \textit{PaHMG2} (\textit{Podan5c}) belonged to a branch that included proteins with a Sterile Alpha Domain (SAM), which is involved in protein-protein and protein-RNA interactions [60]. In some cases, the orthologous relationships, as defined by FUNGIpath, were not in agreement with the HMG-box based phylogeny. The first instance was the mitochondrial protein \textit{mthMG1} (\textit{Podan5d}) and its ortholog \textit{NCU02695} (\textit{Neucr5d}) in \textit{N. crassa}, which failed to group into the same clade within the HMG-UBF_HMG family although FUNGIpath provided a high confidence score for the orthology of \textit{mthMG1} (\textit{Podan5d}) and \textit{NCU02695} (\textit{Neucr5d}) with the HMG-box based phylogeny. Moreover, \textit{mthMG1} (\textit{Podan5d}) and \textit{NCU02695} (\textit{Neucr5d}) have a mitochondrial targeting signal and are characterized by a DUF1898 domain, supporting an orthologous relationship. In another instance, \textit{Nhp6Ap} (\textit{Sacc5a}) and \textit{Nhp6Bp} (\textit{Sacc5b}), two functionally redundant putative inparalogs from \textit{S. cerevisiae} collectively referred to as \textit{Nhp6p} [61,62], were placed in distant clades with \textit{PaHMG6} (\textit{Podan5a}) and \textit{PaHMG4} (\textit{Podan5b}), respectively, and may be outparalogs instead. In contrast, FUNGIpath identified \textit{Nhp6p} (\textit{Sacc5a}, b) as co-orthologs of \textit{PaHMG6} (\textit{Podan5a}). Similarly, \textit{Hmo1p} (\textit{Sacc5d}) from \textit{S. cerevisiae} belonged to the same clade as \textit{PaHMG4} (\textit{Podan5b}), while FUNGIpath identified \textit{Hmo1p} (\textit{Sacc5d}) as an ortholog of \textit{PaHMG6} (\textit{Podan5a}). However, the FUNGIpath confidence score for orthology of \textit{PaHMG6} (\textit{Podan5a}), \textit{Nhp6p} (\textit{Sacc5a}, b), and \textit{Hmo1p} (\textit{Sacc5d}) was low. Further analyses will be necessary to resolve these phylogenetic ambiguities.

\textit{PaHMG9} (\textit{Podan6a}), along with its \textit{N. crassa} ortholog \textit{NCU07568} (\textit{Neucr6a}), were placed between group C and D (Figure 2). Accordingly, CD-Search failed to place \textit{PaHMG9} (\textit{Podan6a}) and \textit{NCU07568} (\textit{Neucr6a}) into either the \textit{MAT_A_HMG} or HMG-UBF groups (Table 1). Interestingly, the \textit{PaHMG9} HMG-box (\textit{Podan6a}) is characterized by an atypical residue [see above section] that is conserved in \textit{NCU07568} (\textit{Neucr6a}). Our phylogenic analysis also placed a \textit{MAT_A_HMG-box} protein from \textit{N. crassa} (\textit{NCU02326}, \textit{Neucr2d}) between group C and D (Figure 2). Previous work reported that \textit{NCU02326} (\textit{Neucr2d}) is related to \textit{SpTeSt1} (\textit{Scho2c}) and is the closest homolog to \textit{NCU09387} (\textit{Neucr2c}) [57]. In agreement with this report, FUNGIpath identified \textit{NCU02326} (\textit{Neucr2d}) as an ortholog of \textit{SpTeSt1} (\textit{Scho2c}, clade D in Figure 2) and an inparalog of \textit{NCU09387} (\textit{Neucr2c}). However, unlike \textit{NCU09387} (\textit{Neucr2c}), \textit{NCU02326} (\textit{Neucr2d}) was not placed within clade \textit{G} with all other \textit{SpTeSt1} orthologs. Such inconsistency is not unprecedented, as there are several evidences in \textit{N. crassa} that effective defense against duplicated sequences prevents the maintenance of closely related paralogs in this species [63]. To independently assess which of the two \textit{N. crassa} inparalogs is conserved in \textit{P. anserina}, we compared the environment of these genes in the two species. The shared syntenic observed upstream and downstream of \textit{PaHMG5} (\textit{Podan2c}) and \textit{NCU09387} (\textit{Neucr2c}) (Figure SIA) confirms that these two genes are orthologs. The absence of conserved organization between the \textit{NCU02326} (\textit{Neucr2d}) locus and its putative counterpart in \textit{P. anserina} indicates that this gene is absent in \textit{P. anserina} (Figure S1B).

Phenotypic analysis of HMG-box deletion strains during vegetative growth

The eight additional HMG-box genes identified in this study were inactivated by targeted gene deletion to assess their role in the life cycle of \textit{P. anserina}. Deletions were verified by Southern blot analysis (Materials and Methods). The eight mutants were examined for growth and for macroscopic mycelium alterations on minimal medium in \textit{mat}+ and \textit{mat}− context. The \textit{APahmg2}, \textit{APahmg6} and \textit{APahmg9} strains displayed reduced growth (Table 2). The eight mutants were also tested for cold- and thermo-sensitive growth at 18°C and 36°C and for sensitivity to caffeine (phosphodiesterase inhibitor), fluoxetine, which acts on omsoregulation, and sodium dodecylsulfate, which acts on the cell membrane, without revealing any additive phenotype.

Microscopic examination of a 4-day-old \textit{APahmg9} thallus revealed that the leading edge (apical and sub-apical hyphae) displayed several developmental processes that typically occur in old hyphae in the wild-type strain. For instance, male gametes (spermatoria) were observed in the sub-apical portion of a \textit{APahmg9} thallus (Figure 3). \textit{P. anserina} differentiates special cell structures dedicated to breach and to exploit solid cellulosic substrates like cellophane [64]. These structures resemble appressoria, the specialized infective structures of fungal plant pathogens. The \textit{P. anserina} appressorium-like structures were identical in wild-type and \textit{APahmg9} strains (Figure 3A). However, they emerged sooner at the leading edge in the mutant strain than in the wild-type strain. The first appressorium-like structures were found at 1.58±0.31 mm and 4.08±0.20 mm from the hyphal tip in the \textit{APahmg9} and wild-type strains, respectively. Finally, the most striking phenotype of the \textit{APahmg9} strain was the dramatic increase in hyphal anastomoses. While few anastomoses are usually
observed in the sub-apical portion of a wild-type thallus (Figure 3B), sub-apical hyphae from the \textit{APahmg9} strain fused with each other, leading to bundles of tightly attached hyphae (Figure 3B). Moreover, apical hyphae often fused with each other in the \textit{APahmg9} strain (Figure 3B). We never observed wild-type apical hypha anastomoses, suggesting that the \textit{APahmg} mutation deregulates cell fusion. We therefore named this gene \textit{KEF1} (KEep-on-Fusing 1). To our knowledge, \textit{KEF1} is the first identified gene to have a repressor function during cell fusion, whereas many genes activate this process in \textit{N. crassa} [65,66] and \textit{P. anserina} [67,68] (reviewed in [69]).

**Phenotypic analysis of HMG-box deletion strains during sexual development**

\textit{P. anserina} is a heterothallic (self-sterile) fungus that has two mating types, \textit{mat}+ and \textit{mat}−. Haploid strains of each mating type initiate the sexual cycle by differentiating male gametes (spermatia) and female organs (protoperistia). A phenotypic analysis of sexual development separately evaluates the male and female fertility of each mutant. The female organ is a multicellular structure comprising protective maternal hyphae and the ascogonium, which contains the female gametic nuclei. The spermata are independent cells that can be collected and used to fertilize any strain of opposite mating type. A pheromone/receptor signaling system allows the ascogonium to recognize and fuse with sexually compatible spermata. Therefore, fertilization can be controlled to initiate the development of the fruit-body. The male gametic nucleus is delivered into the ascogonium, which differentiates into the hymenium. Karyogamy takes place in the hymenium and is immediately followed by meiosis. Subsequently, the haploid nuclei are packed into ascospores. Ultimately, the mature ascospores are forcibly discharged from fruit-bodies (perithecia).

To determine whether HMG-box gene deletions impair sexual reproduction, each mutant strain was tested for male and female fertility in both mating types (\textit{mat}+ and \textit{mat}−). The test for male fertility consisted in fertilizing wild-type female organs with spermata from each HMG-box deletion mutant (Figure 4A). This analysis indicated that all mutants, except \textit{APahmg5}, produced functional spermata. Moreover, fertilized female organs differentiated into mature fruit-bodies producing ascospores, indicating that HMG-box deletions in the male nuclei did not affect any developmental steps in the fertilized female organs. For each mutant, reciprocal crosses with \textit{mat}− and \textit{mat}+ wild-type strains behaved similarly, indicating that the observed phenotype was not dependent on mating type. We further quantified spermata produced by each deletion mutant and measured their activity in fertilization assays (Materials and Methods). Two mutants, \textit{APahmg4} and \textit{Akef1} produced five and seven times more spermata, respectively, than the wild-type strain. Spermata from these two mutants and from other mutants displayed the same fertilization ability as wild-type spermata. The overproduction of spermata observed in \textit{Akef1} was in agreement with microscopic observations, illustrating that even young hyphae produce spermata. The \textit{APahmg5 mat}+ and \textit{APahmg5 mat}− strains were found to produce as many spermata as wild-type strains. Thus, male sterility in these \textit{APahmg5} strains can be attributed either to an inability of spermata to fertilize wild-type protoperistia or to an arrest of perithecial development shortly after fertilization.

To test the female fertility, we examined the formation of fruit-bodies by mycelia from each mutant fertilized with wild-type spermata (Figure 4A). No fruit-bodies were produced on mutant mycelium in crosses involving \textit{APahmg5}, \textit{APahmg6}, \textit{APahmg8}, \textit{Akef1} and \textit{Anthmg1} strains, demonstrating that these mutants were sterile as female partners. In contrast, \textit{APahmg2}, \textit{APahmg3}, \textit{APahmg4} and \textit{APahmg7} strains produced fruit-bodies that developed normally and ejected as many ascospores as fruit-bodies from a wild-type cross. For each mutant, reciprocal crosses with \textit{mat}− and \textit{mat}+ wild-type strains behaved similarly, indicating that the observed phenotype was not dependent on mating type.

We also examined the distribution of perithecia on the mycelium. These assays were based on homozygous crosses and were performed by inoculating a mixture of fragmented \textit{mat}+ and \textit{mat}− mycelia in the center of a plate containing minimum medium under constant light illumination (Figure 4B). In this test, a wild-type strain differentiates fruit-bodies mostly in a ring-like area, which was 1 cm wide and located approximately 1 cm away from the inoculation point [70]. Only \textit{APahmg2} and \textit{APahmg7} differentiated fruit-bodies with the same pattern as the wild-type strain. The alterations were striking for \textit{APahmg4}, since fruit-bodies were distributed on the entire surface of the culture and the ring-like area was no longer visible. The pattern was also different for \textit{APahmg3}, which developed a wider ring-like fruit-body area. Furthermore, we observed that this phenotype was exacerbated by...
the deletion of PaHMG7. This difference was confirmed by quantitative measurement of the ring-like diameter in the ΔPahmg3 ΔPahmg7 double mutant (Table 2). The increase in ring diameter (54.9 mm instead of 53.7 mm) observed in the double mutant suggested that the PaHMG3 and PaHMG7 genes together control the distribution of fruit-bodies. Fruit-bodies were never observed for ΔPahmg5, ΔPahmg8 and Δkef1. The ΔPahmg6 mutant did not produce fruit-bodies as fast as the wild-type strains, but extended incubation revealed that it was weakly female fertile. Fruit-bodies were 50 times less abundant than in a wild-type cross and they displayed an altered shape, with a smaller body and a larger neck than the wild-type fruit-bodies (data not shown). They began to eject ascospores 8 days after fertilization, whereas completion of the sexual process took 4 days in a wild-type cross. Genetic analysis of their progeny confirmed that they were produced by ΔPahmg6 parents and not by contaminating mycelium.
In conclusion, all the eight HMG-box deletion strains analyzed in this study are altered phenotypically. Complementation assays confirmed that the deletion was responsible for the observed phenotype (Materials and Methods). None of the eight HMG-box genes analyzed in this study were essential for viability, although the deletion of three genes (PaHMG2, PaHMG6 and KEF1) resulted in growth alterations. Most HMG-box gene deletions affected sexual reproduction (Table 2). PaHMG2 is the only P. anserina HMG-box gene whose deletion exclusively affected vegetative growth. Deletions of PaHMG3, PaHMG4 and PaHMG6 affected the distribution of fruit-bodies. Most importantly, these deletions failed to affect vegetative growth and fruit-body development. Deletion of PaHMG6, PaHMG8 and KEF1 impaired female fertility, but mutant strains remained male fertile. Strikingly, the ΔPahmg5 mutant exhibits both male and female sterility, an uncommon phenotype in P. anserina that was only reported for mating-type mutants [26].

Heterokaryotic complementation of female sterility caused by HMG-box gene deletions

Microscopic observation of ΔPahmg5, ΔPahmg6, ΔPahmg8 and Δkef1 cultures revealed the presence of protoperithecia, indicating that female sterility was not due to the absence of female reproductive structures. Female sterility of ΔPahmg5, ΔPahmg6, ΔPahmg8 and Δkef1 can be attributed to a defect either in the development of the fruit-body envelope or in the formation of the hymenium, or both. To determine which tissue requires the HMG-box proteins, we performed trikaryon mosaics. In this experiment, mat+ and mat− HMG-box mutant strains were co-cultured with a strain containing a deletion of the mat+ idiomorph...
(Amat) [71]. Except for the loss of the mat+ mating-type gene, the Amat strain had a wild-type genotype. Because the Amat strain lacked the mating-type gene needed for fertilization, it could not participate in sexual reproduction and, thus, could not rescue hymenium defects. However, the Amat strain maintained wild-type vegetative characteristics and could act as a helper strain by complementing maternal defects in a context of sexually compatible mutant strains. Notably, the Amat strain can provide maternal haploid tissues to form the perithecial envelope [72].

Fertile fruit-bodies were observed in the three trikaryotic cultures of APhmg6, APhmg8 and Akef1 (data not shown), indicating that Amat cells surrounding the hymenium of these HMG-box mutants promote the development of functional perithecia. These results demonstrated that PaHMG6, PaHMG8 and KEF1 are required for making the perithecial envelope and that they are dispensable for the hymenium. We also examined the behaviour of the Amat strain in a trikaryon mosaic test. As previously described [44], most Amat cultures died quickly and did not differentiate protoperithecia. A trikaryotic culture involving sexually compatible Amat strains and Amat strain produced perithecia, but no asciopsores were formed (data not shown). These results indicate that the Amat tissue complemented the vegetative requirement for mtHMG1 during protoperithecia formation, while the absence of mtHMG1 in the hymenium led to an arrest of its development. The protein mtHMG1 is therefore required for both protoperithecial and hymenium development.

Trikaryotic cultures of APhmg5 did not produce any perithecia. It is noteworthy that mating-type gene mutations do not affect PaHMG5 transcription [73], thus excluding the possibility that the Amat strain is unable to complement the APhmg5 defects because it is itself affected for PaHMG5 expression. Failure to restore fertility of APhmg5 in the trikaryotic test could reflect the need for this gene in both male and female fertility, which can be difficult to be restored simultaneously. Therefore, we designed an assay to separately evaluate the restoration of female and male fertility of APhmg5. To evaluate whether the female sterility of APhmg5 could be rescued by a Amat PaHMG5+ strain, co-cultures of mat+ APhmg5 and Amat PaHMG5+ strains were used as female partners in crosses fertilized by mat− wild-type spermatia. Conversely, to evaluate whether Amat PaHMG5+ could restore the male competency of APhmg5, cultures of the mat− wild-type strain were fertilized with spermatia issued from a co-culture of mat+ APhmg5 and Amat PaHMG5+ strains (Materials and Methods). Two fruit-bodies were observed on 10 Petri dishes for the female sterility restoration assay, while three fruit-bodies were observed on 10 mat− plates for the male restoration assay. Several thousand perithecia were formed with wild-type strains in similar experiments, indicating that male and female sterility of APhmg5 strain was inefficiently complemented by the Amat PaHMG5+ strain. Taken together, these data are consistent with a requirement of PaHMG5 for fertilization or an early stage of hymenium development. The arrest in the development of the hymenium at an early stage precludes any conclusion on the role of PaHMG5 in the development of the maternal perithecial envelope.

Defects caused by overexpression of PaHMG5

In the complementation assay of the APhmg5 mutant, introduction of the PaHMG5 wild-type allele restored male fertility but transformants remained female sterile (Materials and Methods). Moreover, the transformants displaying the highest efficiency as male partners were vegetatively altered, displaying a flat vegetative mycelium without aerial hyphae. These data suggested that deregulation of PaHMG5 may be detrimental to the fungus. To examine the consequences of unregulated PaHMG5 expression, a plasmid was constructed to express a fusion of PaHMG5 with the Pagpd (glyceraldehyde-3 phosphate-dehydrogenase) promoter and initiation codon [74] (Materials and Methods). When this fusion construct was introduced into protoplasts from the wild-type strain, most of the recovered transformants displayed a flat mycelium. In addition, they exhibited female sterility in crosses with the wild-type strain. Genetic analysis (Materials and Methods) demonstrated that the vegetative defect (flat mycelium) and female sterility consistently co-segregated with the Pagpd:PaHMG5 transgene. These observations strongly suggested that female fertility relies on tight regulation of PaHMG5 expression.

Transcriptional expression of mating-related HMG-box genes and downstream target genes

PaHMG5, PaHMG6, PaHMG8, KEF1 and mtHMG1 [44] are involved in the development of male and female organs, raising the question of their genetic interactions. To assess the relationships between these genes, quantitative real-time RT-PCR (RT-qPCR) was used to examine their expression patterns in APhmg5 (Table S3), APhmg6 (Table S4), APhmg8 (Table S5), Akef1 (Table S6) and Amat (Table S7) strains in a mat+ and mat− context. Genes were defined as up-regulated in the mutant strain if the fold change (FC) was >1, with a p-value of <0.05 (see Materials and Methods for FC computation). On the other hand, genes were defined as downregulated in the mutant strain if 0<FC<1 with a p-value of <0.05. FCS with a 95% confidence interval including the value of 1 were not considered significant [75]. The results are summarized in Figure 5. Most of the deletions had similar effects in mat+ and mat− strains. The few exceptions were statistically non-significant results in mat+ or mat− strains, e.g., mtHMG1 had a significant FC (1.35) in mat+ Akef1 relative to wild-type and a non-significant FC (1.1) in mat− Akef1 (Table S6). A map of the genetic interactions among PaHMG5, PaHMG6, PaHMG8, KEF1 and mtHMG1 was constructed based on the assumption that deletion of a regulatory gene can affect downstream target genes, while non-affected genes are either upstream regulatory genes or genes acting in an independent pathway (Figure 6). Interestingly, PaHMG6, PaHMG8, KEF1 and mtHMG1 appeared to interact and converge on a key regulator, PaHMG5. We detected an effect of mtHMG1 on the transcription of KEF1 and downstream HMG-box genes, suggesting that this protein is localized to the nucleus although it has been shown to be targeted to the mitochondria [44]. Re-examination of its sequence using PSORTII [76] revealed the presence of several monoparate and bipartite nuclear localization signals.

We further examined the transcription levels of mating-type genes and of a selection of specific target genes [73], including the pheromone and pheromone receptor genes, in the different mutant strains (Table S3 to S7 and Figure 5). We found that the transcription of the mating-type genes FMR1 and FPR1 was reduced in APhmg5 and APhmg8 strains. As expected from the cascade shown in Figure 6, FMR1 was downregulated in mat− APhmg6; in contrast, we failed to identify a significant reduction in FPR1 transcription in mat+ APhmg6. However, all tested FPR1 target genes were either up- or downregulated in this latter strain, suggesting that PaHMG6 by-passes FPR1 to control its target genes. Overall, transcription of the mating-type target genes increased in Akef1 strains, in agreement with the repressor effect of KEF1 on mating-type genes. Interestingly, transcription of pheromone receptor genes decreased in female sterile HMG-box mutants, even in the Akef1 strain. This is in agreement with their essential role in female fertility [73].

PaHMG5 appears to be the major regulator of FPR1 and FMR1 mating-type genes in the network presented in Figure 6. This
raises the possibility that constitutive expression of FPR1 and FMR1 could compensate for the absence of PaHMG5 and, thus, rescue sterility of the ΔPahmg5 mutant. Transgenic versions of FMR1 and FPR1 driven by the Angpd constitutive promoter were previously reported to complement loss-of-function of the corresponding gene [77]. Functional Angpd-FMR1 or Angpd-FPR1 transgenes were introduced by genetic crosses in mat− and mat+ ΔPahmg5 mutant strains, respectively. We observed that ΔPahmg5 strains carrying these transgenes remained male and female sterile, indicating that PaHMG5 function is not limited to the transcriptional activation of mating-type genes. It may regulate mating-type target genes as a cofactor of mating-type transcription factors. Alternatively, it may regulate fertility genes that are different from mating-type target genes.

Promoter region analysis of HMG-box genes and target genes of HMG-box transcription factors reveals HMG-box binding sites

The regulatory network shown in Figure 6 consists of a cascade of HMG-box genes, suggesting that each gene may contain a binding site for the upstream regulating HMG-box factor. A search for a conserved binding site using MEME [78] identified a consensus motif (A/G)ACAAAGAA in KEF1, matHMG1, PaHMG5, PaHMG6, and the FMR1 and FPR1 mating-type genes (Figure 7A). This consensus motif is very similar to the common core DNA motif A(A/T)CAA(A/T)G that is recognized by HMG-box transcription factors [79] (reviewed in [2]). The remaining P. anserina HMG-box genes either contained a sequence that displayed some differences to the A(A/T)CAA(A/T)G core sequence (PaHMG2, PaHMG3, PaHMG4, and PaHMG6) (Figure 7A), or they did not contain any related sequence (PaHMG7 and SQR2). Further analyses of mating-type target genes using MEME revealed that the mating pheromone receptor gene (PRE1), alternative oxydase gene (AOX), phospho-eno-l pyruvate kinase gene (PEPCK), Pa_1_24410 and Pa_6_7350 also contained the core HMG-box binding site ACAAAGA (Figure 7A). Interestingly, the two pheromone genes (MFP and MFM) displayed the same conserved core sequence, ATCAAAG. The mating pheromone receptor (PRE2), Pa_4_80, Pa_4_2858 and Pa_5_9770 did not contain the core HMG-box binding site, suggesting that these genes are secondary targets of HMG-box genes. A total of eight genes contained the A(G/A)ACAAAGAA consensus site. The comparison with the distribution of this site in the P. anserina genome indicated that the consensus site is significantly enriched in the selected set of genes examined here (p-value = 0.016) (Materials and Methods).

Electrophoretic mobility shift assays

We further tested the binding of the heterologously expressed His-tagged version of the entire PaHMG5 protein (HMG5His) in

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electrophoretic mobility shift assays (EMSAs) with primers containing the putative binding sites identified above. We first examined the binding of HMG5His to its putative target genes (FMR1, FPR1 and PaHMG8, Figure 6), including PaHMG5. These genes contained the consensus site (A/G)ACAAAGAA. Under the binding conditions used, HMG5His strongly bound to primers containing these sequences and showed decreased binding upon addition of unlabelled competitor (Figure 8A). Moreover, a primer with a scrambled FMR1 sequence did not display any binding of HMG5His, confirming the specificity of the sequence recognition. These experiments support the idea that PaHMG5 directly regulates these target genes as well as its own transcription, as previously demonstrated for SpSte11 [80]. We further examined the affinity of HMG5His for the putative binding sites of the mating-type target genes (Figure S3), which displayed binding sites different from the consensus. Binding of HMG5His to sites that were different from the consensus (A/G)ACAAAGAA was much weaker than to the consensus binding site (see MFM, MFP AOX and PEPCK in Figure S3). The binding to MFM, MFP and PRE1 sites was more carefully investigated (Figure 8B to D). Reciprocal competition of MFM, MFP and PRE1 sites with the FMR1 consensus site confirmed that HMG5His had a greater affinity for the consensus sequence than for the sites of these mating-type target genes. These results indicate that PaHMG5 recognized the sites in the 5’UTR of mating-type target genes, but may require the mating-type transcription factors to increase the efficiency of binding. We also evaluated the affinity of HMG5His for the putative binding sites of the HMG-box genes that are not regulated by PaHMG5 in our proposed network (Figure 6). HMG5His bound strongly to KEF1 and mhMG1 sites, which are identical to the consensus sequence, while binding to PaHMGG2, PaHMGG3, PaHMGG4 and PaHMGG6 sites was much less efficient (Figure S4). Strikingly, the less efficient binding occurred with sites that were different from the consensus by modification of the central part of the site (see PaHMG2, PaHMG3 and PaHMG6 in Figure S4). Reciprocal competition confirmed that HMG5His has much less affinity for the PaHMG2, PaHMG3, PaHMG4 and PaHMG6 sites than for the consensus site (Figure S5). We assume that the binding of HMG5His to these sites corresponded to the recognition of an HMG-box binding site by an HMG-box protein, but this did not demonstrate in vivo regulation by PaHMG5.

Figure 7. Conserved sequences found in the promoter region of HMG-box genes and mating-type target genes. (A) Conserved sequences from a number of HMG-box genes and mating-type target genes identified using MEME program [78]. Position –1 is the first nucleotide upstream of the translation initiation codon. (B) Weblogo of the consensus sequence generated by MEME (assembled from sequences listed in A). (C) The P. anserina consensus sequence aligned with the binding site of HMG-box proteins: MATa-1 (N. crassa) [109], SpSte11 (S. pombe) [30], Prf2 (U. maydis) [38,39] and Mat2 (C. neoformans) [41]. doi:10.1371/journal.pgen.1003642.g007
indicate that PaHMG5 binds most efficiently to sites that are almost identical to those that are recognized by SpSte11, its ortholog in S. pombe.

An analysis of the distribution of the (A/G)ACAAAAAGAA binding site in the P. anserina genome indicated that this motif is preferentially localized on segments of 1000 bp upstream of the predicted translational start sites (p-value of <0.0001) (Materials and Methods). In contrast, a scrambled binding site did not display significant enrichment in these 1000 bp regions corresponding to promoter regions (p-value = 0.08). A total of 502 genes contain the (A/G)ACAAAAAGAA motif, suggesting that up to 5% of P. anserina genes may be directly controlled by MATA_HMG-box transcription factors.

Discussion

Significance and conservation of the HMG-box gene functions in fungi

The data presented here provide new insights into the role and the relationships of HMG-box genes in the fungus P. anserina (Pezizomycotina). We revealed a network of HMG-box genes upstream of the FMR1 and FPR1 mating-type genes, which are themselves HMG-box genes. PaHMG5 plays a central role in this network by regulating mating-type gene expression. We identified SpSte11 as the ortholog of PaHMG5 in S. pombe by different phylogenetic analyses, and we demonstrated that both recognize almost identical binding sites. KEF1 is another important member of the network, acting upstream of PaHMG5 as a repressor. KEF1 also appears as a repressor of hyphal anastomoses, which are dramatically deregulated in a Akef1 strain. Moreover, the control of mating-type target genes by FMR1 and FPR1 was described in a previous report [73], which, together with the results presented here, provides the first exhaustive view of the regulatory circuits upstream and downstream of mating-type genes in a filamentous Ascomycete.

Strikingly, ten of the 12 HMG-box genes identified in the genome of P. anserina control fertility and sexual development. Although two genes (PaHMG2 and PaHMG7) were not directly involved in sexual reproduction, the deletion of PaHMG7 displayed a synergistic effect with the deletion of PaHMG3 on the peritheciun distribution in co-cultures of mat− and mat+ strains. This observation demonstrates that 11 of the HMG-box genes have a direct or indirect function during sexual reproduction. The genome-wide systematic deletion analysis of F. graminearum transcription factors [43] allowed us to compare the resulting phenotypes with those of P. anserina (Table S8). A total of six out of the 11 HMG-box genes deleted from F. graminearum were involved in peritheciun development. One HMG-box gene (FGSG_06760) was not deleted, and the peritheciun distribution on the mycelium was not tested in F. graminearum as described here. Taken together, these data implicate the HMGB superfamily in sexual reproduction in Pezizomycotina, although further analyses in other species should validate this finding.

Comparative analyses of sex regulatory pathways indicate that transcription factors have often evolved to accommodate unique rather than conserved functions, even across closely related lineages [82,83] (reviewed in [84,85]). For example, the pheromone-response pathway is controlled by an HMG-box protein in C. neoformans [41] and a transcription factor distantly related to the homeodomain family in S. cerevisiae [86], providing evidence for a dramatic change in a key regulator in these species, despite the presence of strong effector conservation [41]. Our study revealed that PaHMG5 and SpSte11 [30] are striking exceptions to the unusual plasticity of pathways regulating sex. These two orthologous HMG-box proteins positively regulate mating-type gene transcription in P. anserina and S. pombe. Moreover, the transcription factor analysis published by Son et al [43] suggests that the SpSte11 ortholog in F. graminearum (FGSG_01366, GzHMG010) also shares this conserved function. The phenotype of F. graminearum deleted for GzHMG010 recapitulates the phenotype of mating-type gene deletions (Table S8, see GzHMG010, MAT1-1-1 and MAT1-2-1) and mating-type locus deletion [57]. This feature is expected for genes operating in the same pathway, but molecular analyses will be necessary to determine whether the control of mating-type gene expression by SpSte11 orthologs extends to other fungi. N. crassa contains two co-orthologs of PaHMG5, indicating that control of mating-type gene expression in N. crassa may be more complex than in P. anserina. Based on phylogenetic analyses and analyses of synteny, we identified NCU09387 as the ortholog of PaHMG5, while NCU02326, the paralog of NCU09387, has no counterpart in P. anserina. Mutations of NCU09387 resulted in strains that could mate, but fruit-body development arrested before ascospore formation [53,56]. This phenotype is clearly different from the mating-type deletion, which resulted in a strain that is unable to mate [88]. This observation raises the interesting possibility that NCU02326 may control the expression of mating-type genes during fertilization, while NCU09387 is involved in the regulation of mating-type genes after fertilization.

The HMG-box gene network regulates vegetative and mating-type fertility genes

Sexual reproduction relies on an interplay between vegetative tissues (the mycelium and the maternal hyphae) and sexual tissue (the hymenium). Vegetative tissues critically contribute to optimal conditions for ascogonium formation and to fertility by providing nutrients to growing fruit-bodies [68]. Inside the fruit-body, the hymenium goes through karyogamy and meiosis, and provides signals to the vegetative tissues to sustain nutrient mobilization [26]. This duality raises the question as to whether the alteration
that entailed sterility in the mutant strains affects vegetative tissues, or sexual tissues via mating-type genes. The strains deleted for PaHMG6, PaHMG8 and KEF1 showed female sterility which was restored by complementation with the Anmat strain in trikaryotic mosaic tests. Although PaHMG6, PaHMG8 and KEF1 control transcription of mating-type genes, the trikaryotic test revealed that they also control fertility genes that are independent of mating-type genes but, nevertheless, critical for sexual reproduction.

The regulatory circuit shown in Figure 6 and the complete sterility resulting from the PaHMG5 deletion points to this gene being a major regulator of sexual reproduction in mat+ and mat− strains. We demonstrated that this gene controls mating-type genes and the pheromone/receptor systems. Pheromone and pheromone receptor genes were identified as the most critical mating-type targets for male and female fertility, respectively [73,89]. The deletion of PaHMG5 had a moderate effect on the transcription of mating-type genes (FPR1, FC = 0.25; FMRI, FC = 0.1; Table S3), but strongly reduced the transcription of pheromone genes (MF1, FC = 0.0004; MF2, FC = 0.004; Table S3). This effect on pheromone genes can explain the male sterility phenotype of ΔPaHmg5 strains. Moreover, some genes necessary for the biogenesis of pheromones are regulated by mating-type genes [73], and their transcription may also be decreased in ΔPaHmg5 strains, thereby enhancing male sterility. By contrast, transcription of the PRE2 receptor gene was reduced 4-fold, a value that is unlikely to result in complete female sterility, which was characteristic of the mat+ ΔPaHmg5 strain. This observation suggests that the deletion of PaHMG5 affects the transcription of another target gene that is critical for female fertility. Further experiments will be necessary to identify this target gene and to determine if it is a target of mating-type genes or a vegetative critical fertility gene.

Relationship between the stationary phase and the HMG-box gene network

Several lines of evidence indicate that the competence of P. anserina for sexual reproduction is acquired during the stationary phase. Development of reproductive structures takes place during this phase [90], and the expression of mating-type genes increases up to 1000-fold upon entry into the stationary phase [73]. Our study provides the first evidence of a link between the stationary phase and the HMG-box genes that control the sexual cycle. Deletion of KEF1 resulted in the formation of anastomoses, appressorium-like structures, and spermatia in young hyphae, all hallmarks of the stationary phase. We propose that this gene is a critical repressor of the switch to the stationary phase, which underlies the morphological transitions of this stage. However, this gene only contributed moderately to the increase in mating-type gene expression, as its deletion resulted in a 3-fold increase of FMRI and FPR1 transcription. Other pathways are likely to contribute to the stationary phase and sexual competence. Two MAP kinase pathways, PaMpk2 and PaMpk1, have essential roles in establishing the stationary phase in P. anserina [67,90,91]. No experimental evidence is yet available in P. anserina to support the link between these MAP kinase pathways and HMG-box genes. Exhaustive analyses of MAP kinase pathways in S. cerevisiae and P. pombe revealed numerous connections with HMG-box proteins. For instance, SpSte11 is a direct target of the Spk1 MAP kinase pathway in S. pombe [92], suggesting that PaHMG5 may be a phosphorylation target of the Spk1 ortholog in P. anserina (PaMpk2). Another interesting connection was uncovered in S. cerevisiae between Nhp6p and the Mpk1p (Sk2p) MAP kinase pathway [61], which is orthologous to the PaMpk1 MAP kinase cascade in P. anserina. These connections provide future direction to find the molecular pathways linking the stationary phase and the sexual cycle.

A module of two HMG-box genes involved in sexual development is present in animals and fungi

Sex determination is highly variable, in contrast to other developmental systems that are well conserved through evolution. Even within a single kingdom, studies on common laboratory model organisms reveal that the genetic mechanisms of sex determination bear little, if any, resemblance. Strikingly, PaHMG5 and SpSte11 have conserved their function as mating-type regulators in P. anserina and S. pombe [30], although these two organisms have diverged 550 million years ago [93]. Moreover, the mating-type genes in both fungi are themselves HMG-box genes, thereby defining an HMG-box module that is conserved in both fungi. An HMG-box module involved in sexual reproduction is also present in the basidiomycete fungus U. maydis. The Rop1 protein directly regulates the transcription of prf1, another HMG-box gene [37]. The Prf1 protein in turn induces the expression of mating-type genes [38,39]. HMG-box genes also play a critical role in sex determination in vertebrates. Sox9, the mammalian Y-chromosomal testis-determining HMG-box gene, is an activator of Sox9. Sox9 is also conserved among non-mammalian vertebrate species and has an ancestral and pivotal role in sex determination [2]. The conservation of a similar regulatory HMG-box module in vertebrates and Dikarya reveals a commonality of sex regulation in animals and fungi. Sox and Sox9 are not orthologs of the fungal HMGB module genes. The two modules are thus analogs, not homologs. However, Martin et al [27] noted that the SoxM protein of Phycomyces blakesleeanus was classified within the SOX-TCF_HMG subfamily. This placement is confirmed in the phylogram presented here (Figure 2: SexM, Phylb8). The discovery of a SOX-TCF_HMG-box regulator of mating-type genes in P. blakesleeanus would indicate an ancestral origin for an HMG-box module involved in sex determination in Opisthokonta. Further investigations on HMG-box transcription factors and sex regulation in fungi should provide relevant information about the conservation and evolution of such modules.

Materials and Methods

Strains and media

The genetic and biological features of P. anserina were first described by Rizet and Engelmann [94] and current culture techniques can be found at http://podospora.igmors.u-psud.fr/methods.php. The strains used in this study were all derived from the S strain [95], which was used to determine the P. anserina genome sequence [53].

Identification of HMG-box genes and structural analyses

The P. anserina genome and protein databases are available at http://podospora.igmors.u-psud.fr/. Identification of HMG-box genes was checked using Fungal Transcription Factor (http://ftfd.snu.ac.kr/intro.php) [49] and the Superfamily databases (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html) [50]. For domain identification, Pfam [96] and CD-searches [51] were run on http://pfam.sanger.ac.uk/ and http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, respectively. N. crassa HMG-box genes were obtained from http://www.broadinstitute.org/annotation/ genome/neurospora/MultiHome.html. FUNGIPATH [52] was used to identify systemically orthologous groups of genes (http://embg.igmors.u-psud.fr/fungipath/). Subcellular localization was predicted with PSORTII [76] at http://psort.hgc.jp/form2.html.
Statistical test for HMG-box gene distribution in the \textit{P. anserina} genome

A total of nine HMG-box genes mapped to chromosome I and three HMG-box genes mapped to other chromosomes. Among the nine genes that mapped to chromosome I, three mapped to the mating-type locus and corresponded to \textit{mat}+ and \textit{mat}− idiomorphs (reviewed in [26]). The idiomorph considered for statistical analysis was the \textit{mat}+ idiomorph, which contained one HMGBox gene (\textit{FPR1}). The total number of HMG-box genes on chromosome I was therefore, seven. The sizes of chromosome I to VII were 8,813,526 bp, 5,165,605 bp, 4,712,833 bp, 3,808,397 bp, 4,734,309 bp, 4,264,133 bp and 4,087,213 bp, respectively. The total size of the \textit{P. anserina} genome was 33,686,016 bp. The expected number of HMG-box genes on chromosome I was between two and three. The p-value was calculated on a contingency table http://www.graphpad.com/quickcalcs/contingency1.cfm using Fisher’s exact test (p-value = 0.07 for two genes; p-value = 0.18 for three genes). The expected number of HMG-box genes on chromosome I was not significantly different from seven genes.

Phylogenetic analyses

Sequence acquisition, identification of consensus amino acids and phylogenetic analysis were performed as described previously [27]. The HMG-box domains used to build the phylogenetic tree (Figure 2) and their alignment are in dataset S1 and dataset S2, respectively.

Gene deletion and complementation

To delete the chromosomal copy of the eight HMG-box genes, eight plasmids containing deletion cassettes conferring resistance to hygromycin B (\textit{Hyg}K) were constructed according to the \textit{N. crassa} strategy for high-throughput generation of gene deletion [97] with modifications aimed at minimizing errors in the 5′ and 3′ flanking regions [73] (see Table S9 for primer sequences). The deletion cassette was released from the vector by \textit{AscI} digestion prior to transformation of \textit{APaKu70} protoplasts [98]. This transformation assay consistently yielded a high percentage of transformants with the correct deletion (>90%). One or two transformants obtained from each assay were subjected to Southern blot analysis to confirm the deletion (Figure S2), and one transformant with the expected hybridization pattern was isolated from the progeny. The total number of HMG-box genes obtained from each of the two transformation assays was 90%. One or two transformants with the expected hybridization pattern were selected for further analysis. The eight primary transformants containing corresponding targeted deletions were genetically purified by crossing with a wild-type strain of opposite mating type. This eliminated untransformed nuclei and segregated out the \textit{APaKu70} mutation through its plasmid-based resistance to hygromycin B (\textit{Phleo}R) phenotype. Screening for [\textit{Hyg}K, \textit{Phleo}R] strains allowed the identification of \textit{mat}+ and \textit{mat}− strains containing the HMG-box deletion but lacking \textit{APaKu70}. These strains constituted the stock of the deletion mutant for subsequent studies.

To ensure that the phenotype(s) observed for the HMG-box gene deletion mutants was actually due to inactivation of the relevant gene, the wild-type allele was reintroduced by transformation into the corresponding mutant. Wild-type alleles were obtained by amplifying fragments encompassing the corresponding gene (see Table S10 for primer sequences) and were used directly for co-transformation of the mutant strain with the pPable vector [77], which conferred resistance to plasmid-carrying \textit{PaHMG5} wild-type allele into the \textit{APaHMG5} mutant only rescued the male defect without restoring female fertility, indicating partial complementation.

Measure of spermatium production and activity

Mutants were grown for 7 and 14 days on Petri dishes containing minimal agar medium. Spermatia were recovered by washing the surface of the dish with 1.5 ml of water and were counted with a haemocytometer chamber. A diluted spermatium suspension was used to fertilize a wild-type strain of opposite mating type and perithelia were counted after incubation for 4 days. Typically, 50% to 50% of spermatia from the wild-type strain were fertilizing, giving rise to perithecia. Duplicates were carried out for all strains and the whole experiment was performed twice to confirm the data.

Microscopy

Microscopic observations were made on 4-day-old mycelia growing on cellophane placed on solid M0 medium (minimal medium lacking dextrin as carbon source) in a Petri dish. Small pieces (1 cm²) of cellophane containing mycelium were cut with a scalpel and mounted upside down in water. Pictures were taken with a Leica DMIRE 2 microscope coupled to a 10 MHz Cool SNAP HQ charge-coupled device camera (Roper Instruments). Since penetration of the cellophane through appressorium-like structures occurs perpendicularly to the surface, pictures were obtained at one micrometer increments to capture this process. Stacks of pictures were analyzed with ImageJ (http://rsb.info.nih.gov/ij) and deconvolution was performed with CombineZP (Alan Bradley; alan@micropics.org.uk). Calculations of the mean and the standard deviation of the difference between emergence of appressorium-like structures and the leading edge of the thallus were made using 20 and 10 individual measurements in wild-type and \textit{APaHMG9} strains, respectively.

Construction of the \textit{APaHmg3} \textit{APaHmg7} double mutant

One heterokaryotic \textit{APaHmg3 mat}+/\textit{APaHmg7 mat}− culture was self-crossed, and \textit{mat}+ and \textit{mat}− homokaryotic double mutants were isolated from the progeny. Single and double mutants displayed \textit{[Hyg}K\textit{]} phenotype and were, thus, indistinguishable. Therefore, a search was performed for asci showing first division segregation of the [\textit{Hyg}K] phenotype (i.e., asci with two [\textit{Hyg}K] and two [\textit{Hyg}R] ascospores). Sensitive ascospore-derived cultures from these asci carried wild-type alleles of both genes; hence, resistant ones harbored mutations in both.

Deregulation of the \textit{PaHMG5} gene

The plasmid pBHGST11 contains a fusion of the \textit{P. anserina} \textit{gpd} promoter and initiation codon [74] with the coding phase of \textit{PaHMG5}. The \textit{Pagpd} promoter was a 0.38 kbp fragment obtained from the \textit{Pab} plasmid [77], digested with \textit{NcoI}, treated with Klenow and digested with \textit{XhoI}. The \textit{PaHMG5} sequence was amplified with \textit{Pfu} (Promega) from GA0AB103CF05 [53] using 5PSTE11 and 3HindSTE11 primers (see Table S10 for primer sequences) and digested with \textit{HindIII}. The \textit{Pagpd} promoter and the \textit{PaHMG5} fragment were then ligated into the \textit{HindIII} and \textit{XhoI} sites of plasmid pBCHygros [99] to yield the pBHGST11 plasmid. Sequencing of the entire fusion confirmed that the first Pagpd codon was in frame with \textit{PaHMG5} and that no mutation altered \textit{PaHMG5}. The pBHGST11 plasmid was introduced into \textit{mat}+ wild-type protoplasts and 10 [\textit{Hyg}K] transformants were phenotypically analyzed in a cross with a \textit{mat}− wild-type strain. A total of nine transformants showed a flat and female sterile mycelium, but they were fertile as male partners. To determine
more precisely the phenotypic effects resulting from the integration of the \textit{Pagpd::PaHMG5} fusion, the progeny from three representative transformants were subjected to genetic analysis. Segregation of the \textit{Pagpd::PaHMG5} fusion was scored through the [Hyg\textsuperscript{R}] phenotype. Most unpigmented ascospores did not germinate. Their genotype could nevertheless be deduced from tetrad analysis. For two transformants, the presence of the \textit{Pagpd::PaHMG5} fusion was responsible for an ascospore pigmentation and germination defect (although most unpigmented ascospores did not germinate their genotype could nevertheless be deduced from tetrad analysis). However, pigmented ascospores giving rise to a [Hyg\textsuperscript{R}] mycelium were recovered in the same progeny. These displayed a similar phenotypic vegetative alteration as observed in the primary transformants (flat mycelium and female sterility). In a tetrad corresponding transformant to a different integration site (different second division segregation \% of [Hyg\textsuperscript{R}]/[Hyg\textsuperscript{S}]), the presence of the \textit{Pagpd::PaHMG5} fusion did not affect ascospore pigmentation; instead it conferred the vegetative mycelium defect (flat mycelium and female sterility). These data and conclusions were subsequently confirmed by analyzing second generation progeny which were obtained by crossing the purified [Hyg\textsuperscript{R}] \textit{Pagpd::PaHMG5} bearing transformants with the wild-type strain.

RT-qPCR experiments

Vegetative cultures for RNA preparation were performed on Petri dishes containing minimal medium and covered with a cellophane sheet (Bio Rad Hercules, USA). These cultures were inoculated with nine implants from \textit{mat+}, \textit{mat−} or HMG-box mutants of either mating type. Dishes were placed at 27℃ under constant light (0 h) and were removed from the incubation room at 96 h, at which time \textit{P. anserina} was competent for fertilization [73]. Mycelia were harvested and RNAs were extracted as described previously [73]. Purified RNAs were submitted to an additional DNase digestion in solution and cleaned up once more on RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNAs were reverse transcribed with SuperScript III (Life Technologies) according to manufacturer’s instructions. Each time the expression of an intronless gene was quantified, a non-reverse transcripted (NRT) control was performed for each biological replicate. For genes with introns, all primers were designed against the predicted translational start site of each gene. The full length \textit{PaHMG5} cDNA was amplified by PCR with \textit{Nde1} and \textit{HisBam13} (Table S10) according to manufacturer’s instructions. The FC in the expression of a gene of interest was computed as the normalized relative quantity (RQ) of a cDNA in sample relative to that in the control:

\[ RQ = \frac{\text{FC}}{\text{Efficiency of amplification}} \]

The efficiency of amplification was above 1.8 for all analyzed genes. Genes were defined as downregulated in the mutant strain if 0<FC<1 with a p-value of <0.05. On the other hand, genes were defined as up-regulated in the mutant strain if FC>1, with a p-value of <0.05. FCs with a 95% confidence interval including the value of 1 were not considered significant [75].

Consensus motif search and analysis of HMG-box binding motif distribution

Motif searches were conducted using MEME [http://meme.nbcr.net/meme/cgi-bin/meme.cgi] [78] on segments of 1000 bp upstream of the predicted translational start sites. A group of core sequences including \textit{KEF1}, \textit{mtHMG1}, \textit{PaHMG5}, \textit{PaHMG8}, and \textit{FMR1} and \textit{PPR1} mating-type genes was first analyzed using MEME to identify the consensus HMG-box binding site. Subsequently, each candidate sequence was included with the core sequences for MEME analysis. Segments of 1500 bp upstream of the predicted translational start sites were analyzed when genes yielded no hit using MEME. The number of occurrences of the (A/G)ACAAAGAA binding site was counted for the \textit{P. anserina} genome (n = 1149 in 35,686,016 bp). Assuming a random distribution throughout the genome, the (A/G)ACAAAGAA sequence is expected to occur 0.55 time in the promoter regions of the genes selected for MEME analysis [17,000 bp]. The observed and expected numbers of (A/G)ACAAAGAA sequence were compared on a contingency table [http://www.graphpad.com/quickcalcs/contingency1.cfm] and a p-value was computed using Fisher’s exact test. The (A/G)ACAAAGAA sequence is significantly enriched in the selected set of gene analyzed with MEME (p-value = 0.016). We further analyzed the distribution of the (A/G)ACAAAGAA sequence in the promoter region of all \textit{P. anserina} genes. The number of occurrences of the (A/G)ACAAAGAA sequence was counted on segments of 1000 bp upstream from the predicted translational start site of each \textit{P. anserina} gene (n = 502 in 10,460,890 bp). Assuming a random distribution throughout the genome, the (A/G)ACAAAGAA sequence is expected to occur 339 times in the 10,460,890 bp of the promoter region DNA. Thus, the observed distribution of the (A/G)ACAAAGAA sequence in the promoter regions is significantly different from the random distribution (p-value <0.0001). The scrambled \textit{PaHMG5} binding site [A(G/A)AA-GAAACA] occurred 1390 times in the genome of \textit{P. anserina} and 457 times in the promoter regions. A random distribution of the scrambled site would result in 413 occurrences in promoter regions. Thus, the observed distribution of the (A/G)ACAAAGAA sequence in promoter regions is not significantly different from the random distribution (p-value = 0.08).

Protein expression, purification and electrophoretic mobility shift assays

The full length \textit{PaHMG5} cDNA was amplified by PCR with LA Taq (TaKaRa, Shiga, Japan) from reverse transcribed total RNAs with primers \textit{Nde1} and \textit{HisBam13} (Table S10) according to the manufacturer’s instructions. \textit{HisBam13} was designed to introduce a His\textsubscript{6} tag downstream of the 3’ coding sequence. The PCR products were cloned into the pET28 vector (Novagen) between \textit{Nde1} and \textit{BamHI} restriction sites and inserts were sequenced to identify cDNA without mutations. \textit{E. coli} BL21 (DE3) transformed with the recombinant PET28 vector was grown in 2*YT medium (MP Biomedicals) supplemented with kanamycin at 50 μg/ml. Approximately 800 ml of culture medium was
incubated in a shaker at 200 rpm at 37°C until OD600~0.6~0.8. Protein expression was then induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (Sigma) and the cell culture was further incubated at 15°C overnight. Cells were harvested by centrifugation, resuspended in 40 ml of 20 mM Tris pH9.0, 500 mM NaCl, 5 mM β-mercaptoethanol and protease inhibitor cocktail (Roche), and stored at −20°C. Cell lysis was achieved by sonication, and the cell extract was centrifuged at 20000 x g for 30 min at 4°C. The His-tagged protein from the soluble fraction was purified on a nickel-nitrilotriacetic acid column (Qiagen Inc.) and eluted with an isocratic imidazole gradient, followed by a cation exchange step on a HiTrap Heparim column (GE Healthcare) equilibrated in 20 mM Tris (pH9.0), 300 mM NaCl, 5 mM β-mercaptoethanol, and 5% glycerol. The protein was eluted using a linear salt gradient. The production of recombinant protein was confirmed by SDS-PAGE. Lysis, soluble and some purified fractions were tested by western blot analysis. Proteins were resolved by SDS-PAGE. Lysis, soluble and some purified fractions were tested by western blot analysis. Proteins were resolved by SDS-PAGE.

Complementary primers (Table S14) were annealed to yield double stranded 33 bp oligonucleotides with single base 5’ overhangs consisting of a guanine to promote efficient labeling by T4 polynucleotide kinase [103]. The double stranded oligonucleotides were 5′-end labeled by T4 polynucleotide kinase (Thermo Scientific) and [γ-32P]ATP (222TBq/mmole), according to the manufacturer’s instructions. The probe was purified on MicroSpin G-25 columns (GE Healthcare) and further processed as described in [104].

EMSAs were performed with 340 µg of purified HMG5His in 20 mM Tris (pH9.0), 500 mM NaCl, 5 mM β-mercaptoethanol, 5 mM MgCl2 and 5% glycerol supplemented with 1.25 µg of poly(dI-dC) in a total volume of 11 µL. Samples were incubated for 20 min on ice, labeled probe was added, and incubation was continued for 1 h on ice. For competition experiments, unlabelled double stranded oligonucleotides were added after incubating 20 min with poly(dI-dC) and incubated further for 15 min before adding the labeled probe.

Protein-DNA complexes were separated in polyacrylamide gel (6%) in 0.25 x Tris-borate-EDTA buffer at 200 V per gel for ~80 min. Radioactive probes were visualized using a Typhoon laser scanner (GE Healthcare).

Supporting Information

Dataset S1 HMG-box domains used for Figure 2. (TXT)

Dataset S2 Alignment of HMG-box domains used for Figure 2. (TXT)

Figure S1 Comparative organization around SpeSte11 orthologs in P. anserina and N. crassa. Orthologs were determined using FUNGIpath [52]. The gene names are enclosed in arrowed boxes indicating gene orientation. Orthologous genes are enclosed in boxes of identical color and connected with double arrows. Genes enclosed in white boxes do not have any ortholog in P. anserina or N. crassa. Gene sizes are not to scale. A: synteny in P. anserina and N. crassa for genes upstream and downstream of PaHMG5 and NCU09387. The conserved synteny indicates that PaHMG3 is the ortholog of NCU09387. B: organization of genes upstream and downstream of NCU02326 in N. crassa and search for a synteny in P. anserina. The absence of a conserved synteny confirms that the ortholog of NCU02326 is absent in P. anserina. (TIFF)

Figure S2 Genomic Southern blots of HMG-box mutant strains probed with the hygromycin sequence. 1: DNA from APhahg6 transformants #1 and #2 digested with KpnI. Both transformants displayed the expected pattern for homologous recombination. 2: DNA from APhahg2 transformants #1 and #2 digested with KpnI. Only transformant #2 displayed the expected pattern for homologous recombination. 3: DNA from APhahg5 transformants #1 and #2 digested with EcoRI. Both transformants displayed the expected pattern for homologous recombination. 5: DNA from APhahg6 transformants #1 and #2 digested with EcoRI. Both transformants displayed the expected pattern for homologous recombination. 6: DNA from APhahg4 transformants #1 and #2 digested with EcoRI. Only transformant #1 displayed the expected pattern for homologous recombination. 7: DNA from APhahg7 transformants #1 and #2 digested with EcoRI. This transformant displayed the expected pattern for homologous recombination. 8: DNA from APhahg7 transformants #1 and #2 digested with KpnI. Both transformants displayed the expected pattern for homologous recombination. (TIFF)

Figure S3 Electrophoretic mobility shift assays with PaHMG5 and mating-type target gene oligonucleotides. (A) Interaction of His tagged PaHMG5 (HMG5His) with probes corresponding to mat− mating-type target genes: Pa_1_8290 (FMR), Pa_1_9070 (PRE1) and Pa_1_7350 (7350). The probe is indicated below each panel with the sequence of its core HMG-box binding site. The interaction of HMG5His with probe was analyzed without competitor and in the presence of competitor at 100 fold molar excess. (B) Interaction of His tagged PaHMG5 (HMG5His) with probes corresponding to mat+ mating-type target genes: Pa_2_2310 (MFP), Pa_3_1710 (AOX), Pa_4_3160 (PEPCK) and Pa_1_24410 (24410). Legend as in (A). (TIFF)

Figure S4 Electrophoretic mobility shift assays with PaHMG5 and HMG-box gene oligonucleotides. Interaction of His tagged PaHMG5 (HMG5His) with probes corresponding to mat− mating-type target genes: KeF1, mHMG1, PaHMG2, PaHMG3, PaHMG4 and PaHMG6. Legend as in Figure S3 (A). (TIFF)

Figure S5 Electrophoretic mobility shift reciprocal competition assays with PaHMG5 and HMG-box gene oligonucleotides. Legend as in Figure 8 (B). (A) Interaction of the HMG5His protein with the PaHMG2 probe and reciprocal competition between FMR1 and PaHMG2 oligonucleotides. HMG5His has a greater affinity for FMR1 than for PaHMG2 oligonucleotides, as indicated by the efficient exclusion of FMR1 probe by PaHMG2 competitor. (B) Interaction of the HMG5His protein with the PaHMG3 probe and reciprocal competition between FMR1 and PaHMG3 oligonucleotides. HMG5His has a greater affinity for FMR1 than for PaHMG3 oligonucleotides, as indicated by the efficient exclusion of FMR1 probe by PaHMG3 competitor. (C) Interaction of the HMG5His protein with the PaHMG4 probe and reciprocal competition between FMR1 and PaHMG4 oligonucleotides. HMG5His has a greater affinity for FMR1 than for PaHMG4 oligonucleotides, as indicated by the efficient exclusion of PaHMG4 probe by FMR1 competitor and the inefficient exclusion of FMR1 probe by PaHMG4 competitor.
(D) Interaction of the HMG5His protein with the PaHMG6 probe and reciprocal competition between FMR1 and PaHMG6 oligonucleotides. HMG5His has a greater affinity for FMR1 than for PaHMG6 oligonucleotides, as indicated by the efficient exclusion of PaHMG6 probe by FMR1 competitor and the inefficient exclusion of FMR1 probe by PaHMG6 competitor.

(TIFF)

Table S1 | Color scheme used for Jalview.

(DOC)

Table S2 | Code and accession numbers for proteins shown in Figure 2.

(DOC)

Table S3 | Relative quantification of HMG-box gene and mating-type target gene transcription in \( \text{APa}_{\text{mthmg5}} \) and WT strains.

(DOC)

Table S4 | Relative quantification of HMG-box gene and mating-type target gene transcription in \( \text{APa}_{\text{mthmg6}} \) and WT strains.

(DOC)

Table S5 | Relative quantification of HMG-box gene and mating-type target gene transcription in \( \text{APa}_{\text{mthmg8}} \) and WT strains.

(DOC)

Table S6 | Relative quantification of HMG-box gene and mating-type target gene transcription in \( \text{APa}_{\text{mthmg9}} \) and WT strains.

(DOC)

Table S7 | Relative quantification of HMG-box gene and mating-type target gene transcription in \( \text{Amthmg1} \) and WT strains.

(DOC)

Table S8 | Phenotype of \( F. \) graminearum strains deleted for HMGBox genes (adapted from [43]).

(DOC)

Table S9 | Oligonucleotide primers used for HMG-box gene deletion.

(DOC)

Table S10 | Oligonucleotide primers used for HMG-box gene amplification.

(DOC)

Table S11 | Complementation of \( P. \) anserina mutant strains.

(DOC)

Table S12 | Oligonucleotide primers used for RT-qPCR.

(DOC)

Table S13 | Normalization genes (REF) used for RT-qPCR.

(DOC)

Table S14 | Oligonucleotides primers used for EMSA.

(DOC)

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Author Contributions

Conceived and designed the experiments: RD EC CD HvT. Performed the experiments: JAB EC SB TM NL LPR RD. Analyzed the data: JAB EC SB TM NL RD. Wrote the paper: RD EC.

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