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Structure and Function of the Mating-type Locus in the Homothallic Ascomycete, *Didymella zeae-maydis*§

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Homothallic *Didymella zeae-maydis* undergoes sexual reproduction by selfing. Sequence analysis of the mating type (*MAT*) locus from this fungus revealed that *MAT* carries both *MAT1-1-1* and *MAT1-2-1* genes found in heterothallic Dothideomycetes, separated by ~1.0 kb of noncoding DNA. To understand the mechanistic basis of homothallism in *D. zeae-maydis*, each of the *MAT* genes was deleted and the effects on selfing and on ability to cross in a heterothallic manner were determined. The strain carrying an intact *MAT1-1-1* but defective *MAT1-2-1* gene (*MAT1-1-1*;Δ*MAT1-2-1*) was self-sterile, however strains carrying an intact *MAT1-2-1* but defective *MAT1-1-1* gene (Δ*MAT1-1-1*;*MAT1-2-1*), when selfed, showed delayed production of a few ascospores. Attempts to cross the two *MAT* deletion strains yielded fewer Δ*MAT1-1-1*;*MAT1-2-1* than *MAT1-1-1*;Δ*MAT1-2-1* progeny and very few ascospores overall compared to WT selfs. This study demonstrates that, as in the other homothallic Dothideomycetes, both *MAT* genes are required for full fertility, but that, in contrast to other cases, the presence of a single *MAT1-2-1* gene can induce homothallism, albeit inefficiently, in *D. zeae-maydis*.

Keywords: *Didymella zeae-maydis*, mating-type genes, sexual reproduction, homothallism

Introduction

Mating ability in Ascomycetes is genetically controlled by mating type (*MAT*) genes (Herskowitz, 1988, 1989; Turgeon and Yoder, 2000; Debuchy and Turgeon, 2006; Butler, 2007; Debuchy *et al*., 2010), encoded at a single locus consisting of dissimilar sequences called idiomorphs (*MAT1-1* and *MAT1-2*) (Butler, 2007; Debuchy *et al*., 2010; Debuchy and Turgeon, 2006). All species examined in heterothallic (self-incompatible) Dothideomycetes (e.g., *Alternaria alternata, Cochliobolus* spp., *Mycosphaerella* spp., *Phaeosphaeria nodorum, Stemphylium* spp.) (Arie *et al*., 2000; Waalwijk *et al*., 2002; Bennett *et al*., 2003; Inderbitzin *et al*., 2005; Conde-Ferraez *et al*., 2007; Debuchy *et al*., 2010) show similar *MAT* locus organization in which each idiomorph carries a single gene. *MAT1-2* harbors *MAT1-2-1* encoding a high-mobility-group box (HMG) protein (Bustin, 2001) that is a member of the MATA_HMG subfamily of the HMGB superfamily (Soullier *et al*., 1999). *MAT1-1* harbors *MAT1-1-1* encoding a homolog of the yeast *Saccharomyces cerevisiae* alpha box motif protein (α1), which has been identified as a MATA_HMG subfamily member also (Martin *et al*., 2010). Unlike their heterothallic relatives, homothallic (self-compatible) Dothideomycetes carry both *MAT* idiomorphs found in heterothallic species in a single nucleus, but in contrast to heterothallic counterparts, the structural organization of individual *MAT* genes is unique. Some, such as those from *Cochliobolus luttrellii* and *C. homomorphus* are fused, some are linked (as in *C. kusanoi* and *Stemphylium* spp.), while others are not closely linked (as in *C. cymbopogonis*) (Yun *et al*., 1999; Inderbitzin *et al*., 2005). Detailed investigations of the molecular structures of *MAT* in heterothallic and homothallic *Cochliobolus* (Yun *et al*., 1999) and closely related *Stemphylium* (Inderbitzin *et al*., 2005) have demonstrated that the homothallic reproductive mode evolved from the heterothallic mode, likely from a recombination event within the largely dissimilar *MAT* genes (Yun *et al*., 1999), or by the fusion of an inverted *MAT1-1-1* region to *MAT1-2-1* (Inderbitzin *et al*., 2005).

Since *MAT* genes in filamentous ascomycetes play a central role in sexual reproduction and encode similar transcriptional activators, molecular manipulation can be attempted to determine how well *MAT* sexual development functions are conserved and to clarify whether or not the key molecular differences between homothallic and heterothallic species reside at *MAT*. For *Cochliobolus* spp., *MAT*-deletion analyses confirmed that *MAT* controls not only fertilization but also the subsequent developmental events in both heterothallic and homothallic *Cochliobolus* spp. (Wirsel *et al*., 1996, 1998; Lu *et al*., 2011). This is also true for Sordariomycetes species, (Debuchy *et al*., 2010; Whittle and Johannesson, 2011; Ait Benkhali *et al*., 2013) although the functional requirements of three *MAT1-1* genes for mating vary among species, e.g., between *Gibberella zeae* and *Sordaria macrospora* (Lee *et al*., 2003; Klix *et al*., 2010; Kim *et al*., 2012).

Heterologous expression of a *MAT* gene from a homothallic species (*C. luttrellii* or *C. homomorphus*) in heterothallic *C. heterostrophus* lacking the native *MAT* gene (Yun *et al*., 1999) renders the transgenic strain homothallic, while expression

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of heterothallic *C. heterostrophus MAT* genes in homothallic *MAT*-deleted *C. luttrellii* (Lu *et al*., 2011) renders the transgenic strain heterothallic. The successful alteration of sexual reproductive mode (from heterothallic to homothallic or *vice versa*) in *Cochliobolus* spp., indicates that sexual reproductive mode can be switched bi-directionally by *MAT* manipulation alone in Dothideomycetes. Interestingly, single *C. heterostrophus MAT* genes expressed in homothallic *MAT*-deleted *C. luttrellii* were sufficient to make fertile pseudothecia in a homothallic manner (Lu *et al*., 2011).

Here we describe the molecular structure and function of *MAT* idiomorphs in *Didymella zeae-maydis* (von Arx, 1987) [synonymy: *Peyronellaea zeae-maydis* (Aveskamp *et al*., 2010), *Mycosphaerella zeae-maydis* (Mukunya and Boothroyd, 1973)], a member of the Dothideomycetes, and cause of Yellow Leaf Blight of corn (McFeeley, 1971). Virulence of the fungus to corn is promoted by the host-selective polyketide toxin, PM-toxin (Comstock *et al*., 1973; Yoder, 1973; Kono *et al*., 1983; Danko *et al*., 1984) which is similar in chemical structure and biological activity to the host selective toxin, T-toxin produced by *C. heterostrophus* (Yoder *et al*., 1997; Condon, 2013).

We report that the *D. zeae-maydis MAT* carries both *MAT* genes (*MAT1-1-1* and *MAT1-2-1*) found in heterothallic Dothideomycetes separated by ~1.0 kb of noncoding DNA. We deleted each of these *MAT* genes individually and tested whether or not homothallic *D. zeae-maydis* can be made heterothallic by differential deletion of each *MAT* gene, with intriguing results.

Materials and Methods

Strains, media, mating and single ascospore isolation

D. zeae-maydis wild type strain 3018 was recovered fresh from storage (25% glycerol/-80°C) and grown on potato dextrose agar (PDA) or complete medium (CM) for hyphal growth (Leach *et al*., 1982; Yun *et al*., 1998). Transformants of *D. zeae-maydis* were purified by single ascospore isolation and stored in 25% glycerol at -80°C. All strains were freshly recovered from storage for each experiment. *Escherichia coli* strain DH5α was used for propagation of plasmids. Transformants of *E. coli* were grown on Luria Bertani (LB) agar or liquid medium, supplemented with an appropriate antibiotic (75 μg ampicillin/ml, 25 μg chloramphenicol/ml, or 50 μg kanamycin/ml). *D. zeae-maydis* was selfed as previously described (Mukunya and Boothroyd, 1973; Yun *et al*., 1998).

DNA manipulations, PCR, and sequence analysis

Fungal genomic DNAs were isolated as previously described (Yun *et al*., 1998; Chi *et al*., 2009). Restriction endonuclease digestions, ligations, agarose gel electrophoresis, and gel blot hybridizations were done as described previously (Sambrook and Russell, 2001). PCR primers were dissolved (100 μM) in sterilized water and stored at -20°C. PCR amplification was done as previously described (Yun *et al*., 1999). Nucleotide sequences were assembled and analyzed using a DNASTAR software package (DNAStar Inc., USA). BLAST (Altschul *et al*., 1990) searches were done against the NCBI/GenBank databases.

Plasmid constructions

For plasmid construction, three kinds of cloning vector were used, each containing a gene conferring resistance to ampicillin (*amp*^{R}), chloramphenicol (*chr*^{R}), or kanamycin (*kan*^{R}) (Supplementary data Table S1). The plasmids (pBFDMAT1B and pBCDMAT2H) for deletion of either the *MAT1-1-1* or *MAT1-2-1* gene from *D. zeae-maydis* genome were constructed as follows.

pBFDMAT1B (Supplementary data Fig. S1A): A 3.9 kb *Apa*I-*Xba*I fragment containing the *MAT1-1-1* gene and 77 bp of the *MAT1-2-1* gene was taken from pBCMAT1H (Supplementary data Table S1) and ligated into the *Apa*I-*Xba*I sites of pCRII-1 (Supplementary data Table S1), a derivative of pCRII (Invitrogen, USA, Supplementary data Table S1), containing *kan*^{R} and lacking *Xmn*I and *Sca*I restriction enzyme sites, generating pCRMAT1. To delete a 611 bp *Xmn*I-*Sca*I fragment of the *MAT1-1-1* gene from pCRMAT1, the plasmid was digested with *Xmn*I and *Sca*I, ligated with pBF101 (containing *amp*^{R} and *bsd*^{R} genes, Supplementary data Table S1) that was digested with *Xho*I and treated with Klenow to create blunt ends, then transformed into *E. coli* selecting for amp^R and kan^R. Finally, to remove the portion of the vector corresponding to pCRII-1 from pCRΔMAT1B, the plasmid was digested with *Xho*I, self-ligated, and transformed into *E. coli* DH5α. To delete the *MAT1-1-1* gene from the *D. zeae-maydis* genome, the final construct, pBFDMAT1B (Supplementary data Table S1) was linearized with *Xho*I and transformed into *D. zeae-maydis* (Yun *et al*., 1998).

pBCDMAT2H (Supplementary data Fig. S1B): A 2.6 kb DNA carrying the entire *MAT1-2-1* gene and 319 bp of the *MAT1-1-1* gene was amplified from genomic DNA of wild type *D. zeae-maydis* strain using primers, 4-3/pri5 (5′-AAAGCGACAGACCACCCGAGATAC-3′) and 5-1/pri2 (5′-GAGATGAAGTGGCGAAGCAGA-3′), and cloned into the pCRII vector, resulting in pCRMAT12a (Supplementary data Table S1). A 2.7 kb *Hin*dIII-*Xba*I fragment containing the 2.6 kb amplified fragment was excised from pCRMAT12a and ligated into the *Hin*dIII-*Xba*I site of pUC18, resulting in pUCMAT12a. To delete a 520 bp *Nco*I fragment of the *MAT1-2-1* gene from pUCMAT12a, the plasmid was digested with *Nco*I, blunt-ended with the Klenow fragment, ligated with *Eco*RV-digested pBCATPH(K) (containing *hyg*^{R} and *chr*^{R} genes and lacking the *Kpn*I site, Supplementary data Table S1), then transformed into *E. coli* selecting for amp^R and chr^R. To remove the pUC18 portion of this vector (pUCΔMAT2H), the plasmid was digested with *Kpn*I, self-ligated, and transformed into *E. coli* selecting for amp^S and chr^R. For gene replacement, the final plasmid, pBCDMAT2H (Supplementary data Table S1) was linearized with *Kpn*I and transformed into *D. zeae-maydis* (Yun *et al*., 1998).

Fungal transformation

For transformation of *D. zeae-maydis*, conidia harvested from 7-day-old PDA cultures were inoculated into 100 ml of PD broth at 10^6/ml, and incubated for 20 h at 24°C, after which young mycelia from the culture were distributed to

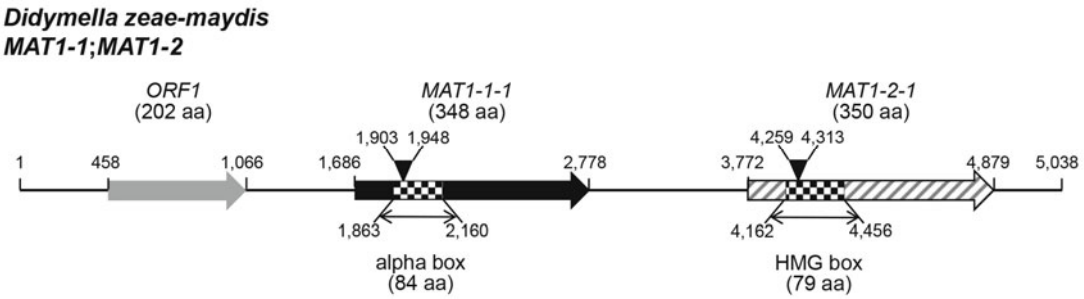

Fig. 1. Organization of *MAT* locus in *D. zeae-maydis*. Thick arrows indicate the locations of the ORFs and their transcription directions. Black boxes, *MAT1-1* ORFs *(MAT1-1-1);* striped boxes, *MAT1-2* ORFs (*MAT1-2-1*); the connecting thin lines, noncoding sequences surrounding the *MAT* genes. The putative DNA binding domains in each *MAT* gene (alpha box in *MAT1-1-1* and HMG box in *MAT1-2-1*) are indicated by checkerboard pattern. The vertical arrowheads indicate the putative introns recognized by comparisons with conserved *MAT* regions in Dothideomycetes. The numbers above or below the ORFs, DNA-binding domains, and introns correspond to nucleotide positions.

two fresh PD broth preparations (100 ml in 300 ml flask) and incubated for an additional 18 h under the same conditions. Mycelia were digested for protoplasting with 10 mg Driselase (Karlan Research Products Corp., USA) and 5 mg Cellulase Onuzuka R-10 (Kinki Yakult Mfg. Co., Ltd., Japan) in 80 ml osmoticum (0.7 M KCl and 0.2 M $CaCl_2$). All other transformation steps using protoplasts were performed as described previously (Yun *et al*., 1998). Two genes, conferring resistance to hygromycin B (*hygB*), or blasticidin S (*bsd*), were used as selectable markers for fungal transformation.

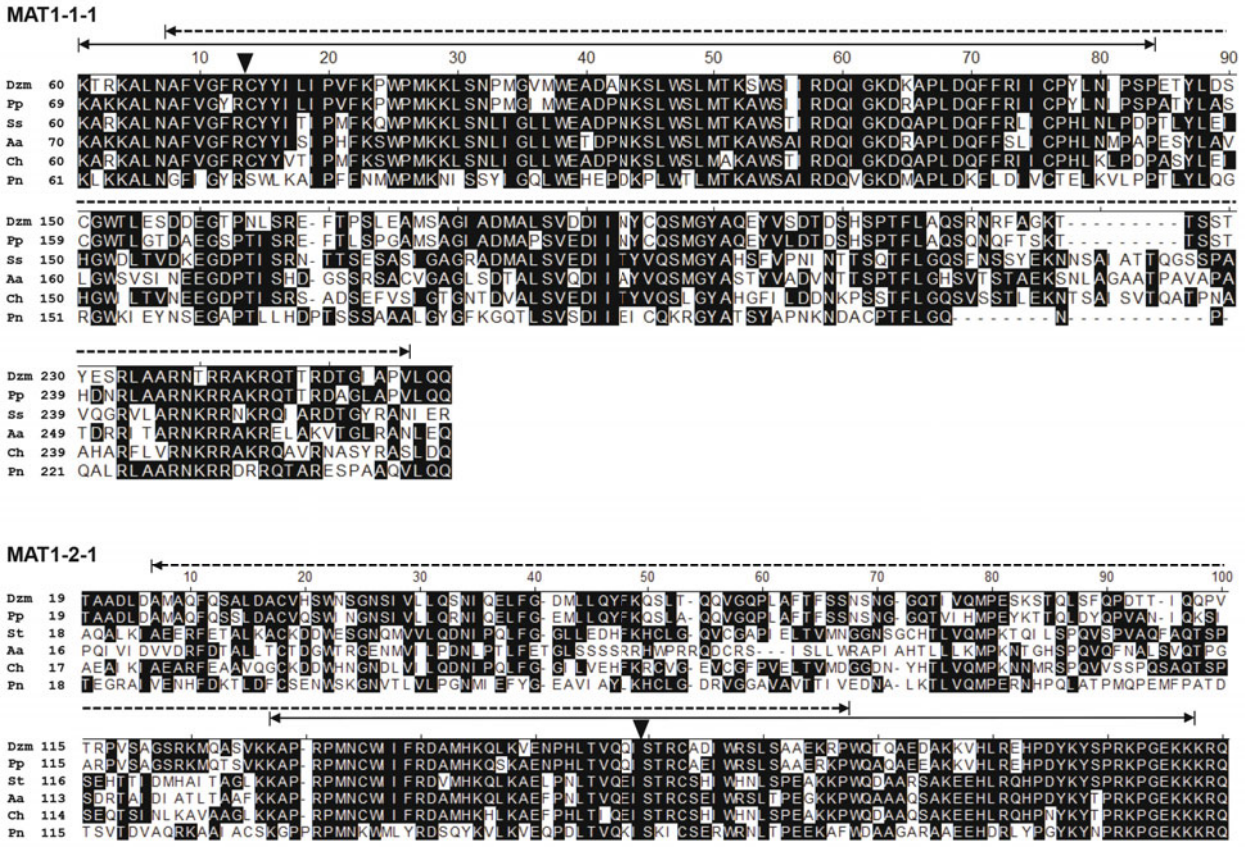

Fig. 2. Partial amino acid sequence alignment of MAT proteins from *D. zeae-maydis* and other Dothideomycetes. The putative DNA binding domain in each MAT proteins (alpha box in MAT1-1-1 and HMG box in MAT1-2-1) is indicated by thin horizontal bar with arrowheads. The *MAT* regions deleted in Fig. 3 are indicated by dashed line with arrowheads. The vertical arrowheads indicate the putative introns shown in Fig. 1. Abbreviation and GenBank accession number: Dzm (*D. zeae-maydis*) MAT1-1-1 (KF443802), Dzm MAT1-2-1 (KF443802), Pp (*Peyronellaea pinodella*) MAT1-1-1 (AER26927), Pp MAT1-2-1 (AER26933), Ss (*Stemphylium solani*) MAT1-1-1 (AAR04452), St (*Stemphylium* sp.) MAT1-2-1 (AAR04475), Aa (*Alternaria alternata*) MAT1-1-1 (BAJ10509), Aa MAT1-2-1 (BAA75903), Ch (*Cochliobolus heterostrophus*) MAT1-1-1 (Q02990), Ch MAT1-2-1 (Q02991), Pn (*Phaeosphaeria nodorum*) MAT1-1-1 (XP_001791062), Pn MAT1-2-1 (AY212019).

Fungal transformants were selected on a regeneration medium containing appropriate antibiotics.

Results

Structure of the *MAT* locus in *D. zeae-maydis*

The *MAT* locus of *D. zeae-maydis* (Fig. 1) was cloned using a combination of PCR amplification, targeted integration, and chromosome walking strategies. First, a 276 bp fragment of the HMG box of *MAT1-2-1* was amplified using the degenerate HMG primers (ChHMG1 and ChHMG2) (Arie *et al*., 1997), then TAIL-PCR (Liu and Whittier, 1995) was performed to obtain sequence beyond the HMG box using combinations of arbitrary and specific primers. For the 5′ flank of the HMG sequence, TP3/ChHMG2 (Arie *et al*., 1997) and TP3/DzHMG4 (5′-TGTTCGCGCAAATGCAC CTTCTT-3′) and for the 3′ flank TP1/ChHMG1 (Arie *et al*., 1997) and TP1/DzHMG5 (5′-AACCCTCACCTCACCGT ACAGCAGAT-3′) were used. Sequencing of the extended 5′ (~1.7 kb) and 3′ (~0.5 kb) flanks of the HMG box revealed the complete *MAT1-2-1* sequence and 320 bp of *MAT1-1-1* DNA separated by ~1.0 kb of non-coding DNA (Fig. 1). To recover the entire *MAT1-1-1* sequence, the *MAT1-2-1* region was deleted and replaced with linearized pBCΔMAT2H (Supplementary data Fig. S1), then a 10 kb *Xho*I DNA fragment 5′ of the vector insertion point (evident in DNA gel blot analysis, not shown), carrying the entire *MAT1-1-1* sequence was recovered from *hygB*^{R} *MAT1-2-1* deletion mutant DzTXΔMAT2-15 (Figs. 2 and 3), using a plasmid rescue procedure (Yun *et al*., 1998).

Sequence assembly (5,038 bp) revealed that the *D. zeae-maydis MAT* locus includes ~1.0 kb of *MAT1-1-1* and *MAT1-2-1,* separated by ~1.0 kb non-coding DNA and the complete 0.6 kb of ORF1, typically found on the 5′ flank of both *MAT* genes of heterothallic Dothideomycetes (Fig. 1, GenBank accession no. KF443802). *MAT1-1-1* encodes a 348 amino acid (aa) protein showing 81.0% amino acid identity to *Peyronellaea pinodella* MAT1-1-1 (accession no. AER26927.1), and 78.6% identity over the alpha box conserved region (84 aa) of *C. heterostrophus* MAT1-1-1 (Turgeon *et al*., 1993) (Figs. 1 and 2). *MAT1-2-1* encodes a 350 aa protein with 80.0% identity to *P. pinodella* MAT1-2-1 (AER26933.1) (Woudenberg *et al*., 2012), and 72.2% identity over the HMG box region (79 aa) of *C. heterostrophus* MAT1-2-1 (Turgeon *et al*., 1993) (Figs. 1 and 2). ORF1 (Wirsel *et al*., 1996) shows 81.0% aa identity to the *Exserohilum monoceras* (*Setosphaeria monoceras*) ORF1 and 69.3% identity to the *C. heterostrophus* ORF1 of unknown function, closely linked to the *MAT* locus (Turgeon *et al*., 1993; Morita *et al*., 2012).

Confirmation of *MAT*- deletions

An internal region of *MAT1-1-1* or *MAT1-2-1* was deleted by targeted gene replacement using linearized pBFDMAT1B or pBCDMAT2H (Supplementary data Fig. S1), respectively (Fig. 3). Desired Δ*MAT1-1-1*;*MAT1-2-1* or *MAT1-1-1*; Δ*MAT1-2-1* strains (designated DzTXΔMAT1 or DzTXΔMAT2, respectively) (Fig. 3A and 3B) were identified by Southern blot analysis (Fig. 3C and 3D). The *MAT1-1-1* probe (2.3 kb *Sph*I fragment from pBCMAT1H) hybridized to a 2.3 kb fragment in wild type and DzTXΔMAT2, and to a 6.3 kb fragment in DzTXΔMAT1, indicating that *MAT1-1-1* was deleted in DzTXΔMAT1 and replaced with the vector, pBF101 (Fig. 3C). The *MAT1-2-1* probe (1.8 kb *Sph*I fragment of pCRMAT12a) hybridized to a 1.8 kb fragment in wild type and DzTXΔMAT1, and to a 6.8 kb fragment in DzTXΔMAT2, indicating *MAT1-2-1* was deleted

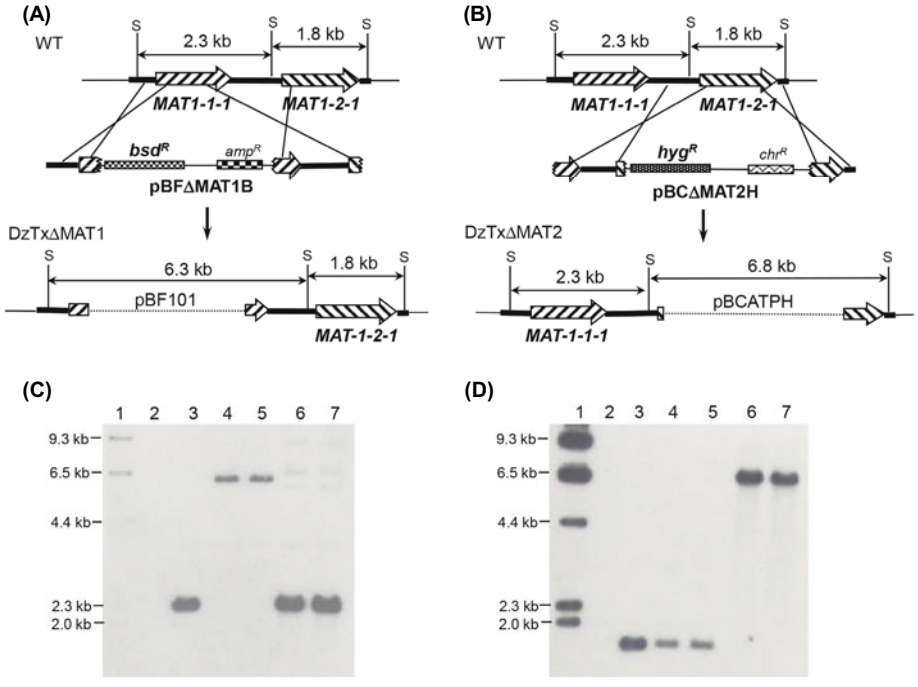

Fig. 3. Targeted gene deletion of each *MAT* gene from the genome of *D. zeae-maydis*. (A-B) Deletion schemes for *MAT1-1-1* and *MAT1-2-1*, respectively. WT: genomic DNA of self-fertile *D. zeae-maydis* 3018 strain, DzTxΔMAT1 and DzTxΔMAT2: genomic DNAs of transgenic strains deleted for an internal region of the *MAT1-1-1* or *MAT1-2-1* gene (see Fig. 2), respectively. (C-D) Southern blot of *Sph*I-digested genomic DNAs from *D. zeae-maydis* strains deleted for either *MAT1-1-1* or *MAT1-2-1*, probed with either *MAT1-1-1* (C) or *MAT1-2-1* (D). Lanes: 1, λ DNA cut with *Hin*dIII; 2, empty; 3, wild type strain; 4, 5, *MAT1-1-1* deletion strains, DzTXΔMAT1-1 and DzTXΔMAT1-4, respectively; 6, 7, *MAT1-2-1* deletion strains DzTXΔMAT2-15 and DzTXΔMAT2-24, respectively. S, *Sph*I cutting site.

in DzTXΔMAT2 and replaced with the vector, pBCATPH (Fig. 3D). In DzTXΔMAT1, a 611 bp region of the *MAT1-1-1* gene including 276 bp of the alpha box sequence (alpha box sequence = 298 bp total) was deleted and replaced with vector pBF101 carrying *bsd*; the *MAT1-2-1* gene was left intact (Figs. 2 and 3A). DzTXΔMAT2 has an intact *MAT1-1-1* gene but carries a 520 bp deletion of the *MAT1-2-1* gene [including 202 bp of the HMG box (276 bp total)], which was replaced with vector pBCATPH carrying *hygB* (Figs. 2 and 3B). No differences were seen between the *MAT*-deletion strains and their WT progenitor (3018) for hyphal growth, pigmentation, conidiation, and PM-toxin production (data not shown).

Mating ability of *D. zeae-maydis* strains deleted for either *MAT1-1-1* or *MAT1-2-1*

A representative of each of the two types of *MAT*-deletion strain was selfed. Unexpectedly, selfs of DzTXΔMAT1 (carrying intact *MAT1-2-1*) still produced some ascospores although shooting of these was delayed 3–4 days compared to that of wild type (WT) selfs and only 1–5% of WT numbers were produced (Table 1). We did not count number of pseudothecia formed as it is very difficult to distinguish these from asexual spore-producing pycnidia. DzTXΔMAT2 (carrying intact *MAT1-1-1*), in contrast, did not shoot ascospores even after more than one month, suggesting it was self-sterile (Table 1).

Crosses between DzTXΔMAT1 (Δ*MAT1-1-1*;*MAT1-2-1*) and DzTXΔMAT2 (*MAT1-1-1*;Δ*MAT1-2-1*) were set up to test if these strains would cross with each other (i.e. in a heterothallic manner). If crossing occurred, the introduced selectable markers, *bsd* and *hygB*, that replaced *MAT1-1-1* and *MAT1-2-1*, respectively, would be expected to segregate in equal numbers since *MAT* is a single locus. Segregation patterns of progeny however, showed off-ratio selectable marker segregation. The overall phenotype was similar to that of selfs of DzTXΔMAT1 i.e., delayed sporulation and few spores. However, both bsd^R and hyg^R progeny were recovered in these crosses (Table 1). In three separate experiments, the former outnumbered the latter ~5:1, 4:1, and 9:1. This suggests that selfing of DzTXΔMAT1 occurred, which would yield bsd^R progeny, and that crossing may have occurred also, which would be expected to yield equal numbers of bsd^R and hyg^R progeny and to add to the overall number of bsd^R progeny. If so, then, in the first experiment, a total of 10 (5 bsd^R + 5 hyg^R) progeny would come from the cross, while 20 came from the self, resulting in a 2:1 ratio of self to outcross. The same type of calculation applies to experiments two (18 from the cross, 15 from the self which is ~ 1:1 self to outcross), and three (8 from the cross, 29 from the self which is ~ 4:1 self to outcross). While these results are variable, they are supported by in replicate experiments.

Table 1. **Ascospore production by selfs and crosses of *D. zeae-maydis* *MAT*-deletion strains**

	Strain	Progeny (no.)[a] of each phenotype	
		bsd^R;$hygB^S$	bsd^S;$hygB^R$
Self[b]	DzTxΔMAT1-1[c]	35	–[e]
	DzTxΔMAT1-4[c]	10	–[e]
	DzTxΔMAT1-8[c]	29	–[e]
	DzTxΔMAT2-15[d]	–[e]	none
	DzTxΔMAT2-24[d]	–[e]	none
Cross[f]	DzTxΔMAT1-1 × DzTxΔMAT2-15	25	5
	DzTxΔMAT1-4 × DzTxΔMAT2-15	33	9
	DzTxΔMAT1-8 × DzTxΔMAT2-15	37	4

[a] Total number per mating plate obtained by the "ascospore shooting" procedure (Yun *et al.*, 1998).
[b] The wild-type 3018 strain produced several thousand ascospores per mating plate.
[c] Each is a transformant carrying the *MAT1-1-1* deletion [DzTxΔMAT1 (bsd^R)].
[d] Each is a transformant carrying the *MAT1-2-1* deletion [DzTxΔMAT2 (hyg^R)].
[e] not expected.
[f] Ascospores were produced in all crosses, but 3-4 days later than in selfs of the wild type strain.

Discussion

Sequenced *MAT* genes from homothallic *D. zeae-maydis* have allowed us to investigate structure of the *MAT* locus and the contribution of individual *MATs* to function in only the second (*Cochliobolus* spp. was the first) homothallic species to be examined in the Dothideomycetes, (Debuchy *et al.*, 2010; Lu *et al.*, 2011). We experimented with the possibility of converting homothallic *D. zeae-maydis* to heterothallism by differential deletion/disruption of each *MAT* gene. From a practical perspective, ability to segregate in a heterothallic manner would facilitate segregation analyses.

The *MAT1-1-1*;Δ*MAT1-2-1* strains, when selfed were completely sterile, suggesting that the function of the MAT1-2-1 protein is essential. In contrast, selfs of the Δ*MAT1-1-1*; *MAT1-2-1* strain produced some ascospores although their appearance was delayed and the numbers of ascospores were much lower than those from a self of WT. This suggests that the function of the MAT1-1-1 HMG domain (alpha box domain) might be redundant with one or more of the other HMG proteins in the genome (Wik *et al.*, 2008; Ait Benkhali *et al.*, 2013) or that the deletion we made resulted in a leaky phenotype. Ability to self of a strain with only a single *MAT* gene is reminiscent of our finding that homothallic *C. luttrellii MAT*-deletion strains carrying only a single heterologous *MAT* gene from the heterothallic *C. heterostrophus* are capable of producing fertile pseudothecia. The size of these and their fertility was lower (4–15%) than those of the WT strain (Lu *et al.*, 2011). Note that the *MAT*-deletion and single *MAT* selfing experiments with *D. zeae-aydis* are with native genes, while the *Cochliobolus* experiments are with heterothallic *C. heterostrophus MAT* genes in homothallic *C. luttrellii*. *MAT*-deleted *C. luttrellii* is completely sterile. Also, in the *C. luttrellii* case large numbers of pseudothecia that were formed were smaller in size than those from WT selfs, while with *D. zeae-maydis*, we could not easily distinguish pseudothecia from pycnidia, and therefore were unable to make this type of comparison. Maintenance of only one type of native *MAT* gene in the homothallic Sordariomycete species *Gibberella zeae* is not sufficient to induce homothallism (Lee *et al.*, 2003).

Other possible reasons for the phenotype of selfs in *D. zeae-maydis* include the possibility that deletion of 611 bp of *MAT1-1-1* DNA in the Δ*MAT1-1-1*;*MAT1-2-1* strain (Fig. 2) did not eliminate all critical regions of *MAT1-1-1*.

However, the *MAT1-1-1* deleted region in the Δ*MAT1-1-1*; *MAT1-2-1* strain includes 77 of the 84 aa (~92%) of the critical DNA binding domain (HMG or alpha box) (Fig. 2), while the completely sterile *MAT1-1-1*;Δ*MAT1-2-1* strain carries a 520 bp deletion of *MAT1-2-1* DNA which includes 50 of the 79 amino acids (~63% only) of the other HMG domain, but is enough to destroy the function (Fig. 2). It is also possible that the MAT1-2-1 protein is more important for function than MAT1-1-1 protein for either initiating or completing meiosis. To investigate the various possibilities, it will be necessary to check *MAT* transcription. If the Δ*MAT1-1-1*;*MAT1-2-1* strain still produces a transcript, we will need to re-make a mutant, this time with the entire *MAT1-1-1* region deleted. If selfs do not shoot ascospores, this supports the possibility that residual MAT activity was the cause of our original phenotype.

The 5:1 bsd^R:$hygB^R$ ratio of ascospore production observed in crosses between the two *MAT*-deletion strains could be explained in several ways. Firstly, perhaps, as we hoped, crossing between the bsd^R, Δ*MAT1-1-1*;*MAT1-2-1* and hyg^R, *MAT1-1-1*;Δ*MAT1-2-1* strains did occur in addition to selfing of bsd^R, Δ*MAT1-1-1*;*MAT1-2-1*. Alternatively, perhaps something produced by the bsd^R, Δ*MAT1-1-1*;*MAT1-2-1* strain promoted selfing of the hyg^R, *MAT1-1-1*;Δ*MAT1-2-1* strain. Unlike heterothallic haploid strains, both *MAT* transcripts might be required to be in a single nucleus of a haploid homothallic cell for further development (e.g. formation of dikaryotic cells or nuclear fusion) for meiosis to occur. If this is true, nuclei producing only one intact *MAT* transcript would not undergo meiosis, therefore, no ascospores would be produced.

An alternative strategy to make a homothallic fungus heterothallic could be to delete the entire *MAT* region from a wild type homothallic strain, add back, separately, a wild type copy of each *MAT* idiomorph of the homothallic strain, or from a heterothallic species, then attempt crosses. This strategy has been proven to work well in homothallic *C. luttrellii* (Lu *et al.*, 2011). In addition, it is necessary to determine if the *MAT* deletion strains of *D. zeae-maydis* carrying a single *MAT* gene have the capability to outcross with their wild-type progenitor carrying both *MAT1-1* and *MAT1-2* idiomorphs, as has been shown for homothallic *C. luttrellii* and *G. zeae* (Lee *et al.*, 2003; Lu *et al.*, 2011).

In conclusion, both *MAT1-1-1* and *MAT1-2-1* genes play important roles in controlling self-fertility in *D. zeae-maydis* through functions conserved among homothallic as well as heterothallic ascomycetes, but the contribution of each MAT protein towards homothallism may be not the same, which may reflect the unique homothallic life style of the Dothideomycetes compared to that of the Sordariomycetes.

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