A Cytosine Methyltransferase Homologue Is Essential for Sexual Development in *Aspergillus nidulans*

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Abstract

**Background:** The genome defense processes RIP (repeat-induced point mutation) in the filamentous fungus *Neurospora crassa*, and MIP (methylation induced premeiotically) in the fungus *Ascobolus immersus* depend on proteins with DNA methyltransferase (DMT) domains. Nevertheless, these proteins, RID and Masc1, respectively, have not been demonstrated to have DMT activity. We discovered a close homologue in *Aspergillus nidulans*, a fungus thought to have no methylation and no genome defense system comparable to RIP or MIP.

**Principal Findings:** We report the cloning and characterization of the DNA methyltransferase homologue A (*dmtA*) gene from *Aspergillus nidulans*. We found that the *dmtA* locus encodes both a sense (*dmtA*) and an anti-sense transcript (*tmdA*). Both transcripts are expressed in vegetative, conidial and sexual tissues. We determined that *dmtA*, but not *tmdA*, is required for early sexual development and formation of viable ascospores. We also tested if DNA methylation accumulated in any of the *dmtA/tmdA* mutants we constructed, and found that in both asexual and sexual tissues, these mutants, just like wild-type strains, appear devoid of DNA methylation.

**Conclusions/Significance:** Our results demonstrate that a DMT homologue closely related to proteins implicated in RIP and MIP has an essential developmental function in a fungus that appears to lack both DNA methylation and RIP or MIP. It remains formally possible that DmtA is a bona fide DMT, responsible for trace, undetected DNA methylation that is restricted to a few cells or transient but our work supports the idea that the DMT domain present in the RID/Masc1/DmtA family has a previously undescribed function.

Introduction

DNA methylation is essential for normal development and differentiation of plants and mammals [1–11]. Fungi like *Neurospora crassa* show substantial DNA methylation despite the fact that the process in this organism is dispensable [12,13]. In contrast, several well-studied organisms completely lack DNA methylation (e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Caenorhabditis elegans*), while in others (e.g., *Drosophila melanogaster* and *Aspergillus flavus*) very little methylation has been reported [14–18], and its significance remains controversial [19].

Although extensively studied, much remains to be learned about the biology and relationship of eukaryotic DNA methyltransferases (DMTs). Putative and established eukaryotic DMTs can be separated into five families [11,20]: 1) the DNMT1 or “maintenance DMT” family (e.g., mammalian Dnmt1 and plant MET1); 2) the DNMT3 or “de novo DMT” family (e.g., mammalian Dnmt3a and Dnmt3b and the plant DRMs); 3) the plant-specific chromomethylase (CMT) family (e.g., Arabidopsis CMT3); 4) the fungal-specific DMT-like family (e.g., *Ascobolus immersus* Masc1 and *Neurospora* RID); and 5) the DNMT2 family. Some fungal DMTs, like *Neurospora* DIM-2 and *Ascobolus* Masc2, do not fit well into any of the above-mentioned groups, but are regarded as highly divergent members of the DNMT1 family; alternatively, they may constitute a sixth, fungal-specific family [11].

It is important to note that proteins that are similar to DMTs in their primary structure (i.e., putative DMTs) may not be true DMTs. This appears to be the case for members of the DNMT2 family that have been recently demonstrated to have tRNA<sup>ASP</sup>, methylation activity [19]. Similarly, DMTase activity has not been demonstrated for either RID [20] or Masc1 [21], putative DMTs associated with RIP (Repeat-Induced Point Mutation) in *Neurospora* and the related process, MIP (Methylation Induced Premieotically), in *Ascobolus*, respectively. RIP, the first eukaryotic genome defense system discovered [22–26] alters DNA duplications during the sexual phase of the life cycle by introducing C:G to T:A transition mutations in both copies of the duplication [27,28]. DNA sequences that have been subjected to RIP are usually, but not invariably, methylated [23–25]. The precise relationship between RIP and DNA methylation remains unknown, but it seems likely that RID is responsible for cytosine methylation and/or deamination, resulting in the observed transition mutations.
[20,26,29–31]. RIP is abolished in homozygous crosses with rid mutants [20].

As in Neurospora, the haploid parental genomes of A. immersus are scanned for DNA duplications after fertilization but before karyogamy. Unlike the situation in Neurospora, however, duplicated sequences are subjected to DNA methylation only; mutations do not occur [21,32–34]. Interestingly, the Ascosbolus RID orthologue, Masc1, is essential for normal sexual development. This is in contrast to homozygous rid crosses, which are completely fertile [20]. It is impossible to test the involvement of Masc1 in MIP in crosses homozygous for mutations in Masc1, is essential for normal sexual development. This is in a phage Lambda ZAP cDNA library (as described in Results) and contains the 1989 bp tmdA cDNA (coordinates 4503 to 2433, Figure 1). Plasmid pDLAM002 was constructed by replacing the 694 bp HindIII-EcoRV (coordinates 3224 to 3999) region of dmtA/tmdA of pDLAM001 with the ~1.8 kbp Nod-Smal fragment containing the argB' from pDC1 after subjecting HindIII-EcoRV-digested pDLAM001 to an end-filling reaction. The dmtA and argB genes have opposite directions of transcription and the region deleted contains the predicted catalytic site of the putative DNA methyltransferase. Plasmids pDLAM004 and pDLAM005 contain the 3224 bp EcoRI-HindIII fragment of dmtA (coordinates 1 to 3224) and 2972 bp HindIII-EcoRI fragment of dmtA (coordinates 3224 to 6195), respectively. Insertion of the 2972 bp HindIII-EcoRI fragment of dmtA/tmdA (coordinates 3224 to 6195) into the HindIII-EcoRI sites of pK19 [38] yielded pDLAM013. Inverse PCR with ODLAM069, which introduces a BamHI site, and ODLAM070 as primers and pDLAM013 as the substrate, followed by self-ligation resulted in pDLAM014. Plasmid pDLAM018 was constructed by inserting the ~1.8 kbp BamHI-Xhol fragment containing the argB' gene from pDC1 into the BamHI-SalI sites of pDLAM014, resulting in a 646 bp deletion (coordinates 4462 to 5107) of the tmdA promoter. Insertion of the 1226 bp ClaI-HindIII dmtA/tmdA fragment (coordinates 1998 to 3224) from pDLAM004 into the ClaI-HindIII sites of pDLAM005 yielded pDLAM039, which carries the 4197 bp ClaI-EcoRI dmtA/tmdA fragment (coordinates 1998 to 6195) inserted into the ClaI-EcoRI sites of pBluescript II KS(+) [39] to direct transcription of the nica promoter [39] to direct transcription of dmtA. These constructs carried the entire nica-tmdA intergenic region.

### Materials and Methods

**Bacterial strains and plasmids construction**

*Escherichia coli* K12 XL1-Blue MR (Stratagene, La Jolla, CA, USA) was the host for most plasmid DNA. When non-methylated DNA was needed for enzyme digestions, either GM2163 or JM110 [35] were used. Plasmid pDC1 was described in Aramayo et al. [36]. Plasmid pRB2 was provided by Thomas H. Adams (Monsanto, St. Louis, MO). Oligonucleotides used in this study are described in Table 1.

Plasmids were constructed by standard procedures [37]. The EcoRI site 2432 bp upstream of the predicted translation initiation signal (ATG) of the DmtA polypeptide was arbitrarily defined as position 1 of the dmtA/tmdA locus (see Figure 1). To obtain plasmid pMF156, we used the sequence information provided by the EST sequences identified in the University of Oklahoma *A. nidulans* cDNA database to design oligonucleotides (AND1 and AND2, Table 1) that were used to amplify a genomic DNA fragment from a wild-type *A. nidulans* strain (FGSC 4, Table 2). This amplified fragment was inserted into the TA-cloning vector pCR2.1 (Invitrogen) to yield pMF156. Plasmid pDLAM001 was obtained from a phage Lambda ZAP cDNA library (as described in Results) and contains the 1989 bp tmdA cDNA (coordinates 4503 to 2433, Figure 1). Plasmid pDLAM002 was constructed by replacing the 694 bp HindIII-EcoRV (coordinates 3224 to 3999) region of dmtA/tmdA of pDLAM001 with the ~1.8 kbp Nod-Smal fragment containing the argB' from pDC1 after subjecting HindIII-EcoRV-digested pDLAM001 to an end-filling reaction. The dmtA and argB genes have opposite directions of transcription and the region deleted contains the predicted catalytic site of the putative DNA methyltransferase. Plasmids pDLAM004 and pDLAM005 contain the 3224 bp EcoRI-HindIII fragment of dmtA (coordinates 1 to 3224) and 2972 bp HindIII-EcoRI fragment of dmtA (coordinates 3224 to 6195), respectively. Insertion of the 2972 bp HindIII-EcoRI fragment of dmtA/tmdA (coordinates 3224 to 6195) into the HindIII-EcoRI sites of pK19 [38] yielded pDLAM013. Inverse PCR with ODLAM069, which introduces a BamHI site, and ODLAM070 as primers and pDLAM013 as the substrate, followed by self-ligation resulted in pDLAM014. Plasmid pDLAM018 was constructed by inserting the ~1.8 kbp BamHI-Xhol fragment containing the argB' gene from pDC1 into the BamHI-SalI sites of pDLAM014, resulting in a 646 bp deletion (coordinates 4462 to 5107) of the tmdA promoter. Insertion of the 1226 bp ClaI-HindIII dmtA/tmdA fragment (coordinates 1998 to 3224) from pDLAM004 into the ClaI-HindIII sites of pDLAM005 yielded pDLAM039, which carries the 4197 bp ClaI-EcoRI dmtA/tmdA fragment (coordinates 1998 to 6195) inserted into the ClaI-EcoRI sites of pBluescript II KS(+) [39] to direct transcription of the nica promoter [39] to direct transcription of dmtA. These constructs carried the entire nica-tmdA intergenic region.

### Table 1. Oligonucleotides used in this study

| Name  | Sequence                                                                 |
|-------|--------------------------------------------------------------------------|
| AND1  | 5’-(4110)-GGCCGTGGGGCGACAATTATCACCTCCTCT-(4136)-3’                      |
| AND2  | 5’-(4334)-TCATTGTTTCTGCGAATTCGAGTCC-(4308)-3’                          |
| ODLAM008 | 5’-(2957)-CAAGACGCCACCAGAAGAT-(2976)-3’                               |
| ODLAM009 | 5’-(4229)-CTCTTTACAGTCTCTTCCATC-(4210)-3’                             |
| ODLAM069 | 5’-(4461)-GAGGCTCTTCTCCGTTGAGCC-(4442)-3’                             |
| ODLAM070 | 5’-(5045)-CAGGTCCTCGGCAAATCTCC-(5064)-3’                               |
| ODLAM087 | 5’-(3234)-AATGACCTCTTGCTTCTCCTGTA-(3257)-3’                           |
| ODLAM088 | 5’-(4547)-CAATCAACCGCTTTACAGACAGC-(4524)-3’                           |

Numbers in parentheses denote dmtA/tmdA coordinates with respect to the EcoRI site 2432 bp upstream of the predicted translation initiation codon (ATG) of DmtA. BamHI, Xhol, and KpnI sites in ODLAM069, ODLAM087 and ODLAM088, respectively, are underlined.

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**Aspergillus strains and culture conditions**

Strains of *A. nidulans* are described in Table 2. Standard conditions were used to maintain and grow cultures, and fungal transformations, fertility tests and genetic crosses were performed according to published protocols [40–43]. Minimal medium was prepared as described (PONTECORVO et al. [53,55]) with minor modifications (6 g/L of NaNO₃). Self-fermentation assays were performed by inoculating ~10⁶ conidia onto properly supplemented solid minimal medium (40 ml/Petri dish). To induce sexual development, we restricted air and light for 24 h after inoculation [44]. Petri dishes were incubated in the dark at 37°C for an additional 20 days, after which sexual structures (cleistothecia) could readily be observed.

**DNA isolation**

DNA extractions from *A. nidulans* were performed as described previously for *N. crassa* [45]. Procedures for Southern blot analysis, and other nucleic acid manipulations were as described [36,45,46].
Total RNA was extracted from mycelia using Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted following the manufacturer's protocol. The resulting RNA was subjected to an additional LiCl precipitation purification step to remove DNA, which consisted of adjusting the final LiCl concentration of the aqueous RNA solution to 2M (from 10M LiCl stock solution). Samples were then incubated at 68°C for 10 min (or until the LiCl dissolves and the cloudiness of the samples disappears), and at 5°C for no less than 6 hours. Samples were then centrifuged for 30 min at room temperature. The resulting RNA pellet was then washed with 70% Ethanol and re-dissolved in water.

Strand-specific Reverse Transcribed-PCR (SSRT-PCR)

For first strand synthesis, 0.1 mg of RNA was mixed with 10 pmoles of each ODLAM008 (tmdA) or ODLAM009 (dmtA) (Table 1) to prime tmdA or dmtA transcripts, respectively, in a 10 μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 10 μM DTT, 75 mM KCl, 6 mM MgCl₂, and 1 mM dNTPs.

Figure 1. Molecular structure of the dmtA/tmdA chromosomal locus. The positions of the main restriction sites present in the region as well as the names, location and orientation of the oligonucleotides used are indicated above the DNA sequence. The DNA sequence of the oligonucleotides used is underlined. The 5'- and 3'-ends of the sequenced cDNAs corresponding to the tmdA transcript along with the dmtA intron (lower case) and the predicted dmtA intron, are indicated above the DNA sequence. The region from HindIII (coordinate 3224) to EcoRV (coordinate 3999) was deleted to generate the J3(3224–3999)dmtA/tmdA::argB⁺ allele. Similarly, the sequence from the 5'-most base of oligonucleotide ODLAM069 (coordinate 4462) to SalI (coordinate 5107) was deleted to generate the J4(4462–5107)dmtA/tmdA::argB⁺ allele. The region from ODLAM087 to ODLAM088 was fused to the niiA promoter to generate the niiA(p)::dmtA[2334–4547]::argB⁺ allele. See text for details.

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Results

GenBank accession numbers

The combined sequence of the dmtA/tmdA inserts contained in pDLAM004 and pDLAM005 was deposited as GenBank accession number AF428247. Accession numbers for predicted fungal DmtAs are: A. oryzae (AAE61916); A. fumigatus (XP_747703); A. terreus (XP_001209776); G. clavatus (XP_001239116); U. sciamorpha (UREG_03572.1, Broad Institute); N. crassa (XP_001222613); G. zeae (XP_001239116); N. haematococca (e.g.wl.11744.1, DOE); A. niger (NM_001239116); F. semitectum (NM_001239116); M. oryzae (NM_001239116); and A. immersus (AAC49849).

Treatment of mycelia with 5-azacytidine (5-AC)

Conidia were propagated in liquid medium containing different concentrations of 5-AC for 72 hr essentially as described previously by Tamace et al. [47]. Conidiation of the resulting mycelial mass was induced as described [46] and the resulting conidia were diluted and spread onto Petri plates. The frequency of “fluffy” phenotypes were induced as described [46] and the resulting conidia were diluted and spread onto Petri plates. The frequency of “fluffy” phenotypes obtained from the different strains tested was then determined among the different 5-AC concentrations tested.

GenBank accession numbers

Table 2. *Aspergillus nidulans* strains used in this study

| Name* | Genotypeb, c | Origin* |
|-------|-------------|--------|
| FGSC 4 | FGSC* | FGSC* |
| FGSC A237 | pabaA1, yA2; veA1, trpC801 | FGSC* |
| FGSC A851 | pabaA1, yA2; ΔargB::trpC; veA1, trpC801 | FGSC* |
| PW1 | b1A1; argB2; methG1; veA1 | P. Weglenski. Department of Genetics. Warsaw University, Poland |
| DLAN2 | pabaA1, yA2, Δ[3224–3999]dmtA::tmdA::argB::trpC::veA1, trpC801 | FGSC A851 transformed with pDLAM002 |
| DLAN3 | pabaA1, yA2, Δ[4462–5107]dmtA::argB::trpC::veA1, trpC801 | FGSC A851 transformed with pDLAM018 |
| DLAN4 | pabaA1, yA2, ΔargB::trpC::veA1, trpC801; n/a::dmtA::[2334–4547]argB::[ectopic] | FGSC A851 transformed with pDLAM044 |
| DLAN5 | b1A1; methG1; veA1 | Cross of FGSC A237 with PW1 |
| DLAN6 | pabaA1, yA2, Δ[3224–3999]dmtA::tmdA::argB::veA1 | Progeny from DLAN2 X DLAN5 |
| DLAN7 | pabaA1, yA2, Δ[4462–5107]dmtA::tmdA::argB::veA1 | Progeny from DLAN3 X DLAN5 |
| DLAN8 | pabaA1, yA2; veA1 | Progeny from FGSC A851 X PW1 |

aDLAN indicate strains constructed for this study by Dong W. Lee.

bAllele numbers or designations are: arginine requirement-Δ (orotidine transcarbamylase), argB; biotin requirement-Δ (biotin synthetase), biA1; DNA methyltransferase-like-Δ (cystathionine-β-synthase), methG1; dmtA (DNA methyltransferase-like-A anti-sense, non-coding transcript), tmdA (DNA methyltransferase-like-A, ΔmthG1; methionine requirement-Δ (cystathionine-β-synthase), methG1; DmtA/tmdA (dmtA/tmdA thryltransferase-like), methG1; DmtA/tmdA (dmtA/tmdA anthranilate synthetase); phosphorybosylanthranilate isomerase), trpC801; veA1, yellow conidia-A2 (bacase-I), yA2.

cConstruction of the different plasmids is described in Materials and Methods.

*DGSC, indicates strains acquired from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City.

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The dmtA/tmdA chromosomal region contains one large open reading frame (dmtA ORF, AN6638; 9 bp ORF, predicted translation initiation signal at coordinate 2433 in Figure 1) interrupted by a single 56 bp intron with canonical 5’- and 3’-consensus splice sites (positions 3851 and 3906, respectively). Conceptual translation of the dmtA ORF predicts a 615 amino acid (aa) polypeptide. Interestingly, analysis of the sequence corresponding to the dmtA cDNA revealed the presence of an 82 bp intron with canonical 5’- and 3’-consensus splice sites, at positions 3280 and 3361, respectively. No long ORFs were found in the tmdA cDNA when searching with a window of 100 aa suggesting that the tmdA transcript is non-coding RNA.

BlastP searches [49] with the predicted DmtA sequence as bait revealed similarities with the group of DMT homologues found to date only in fungi and predominantly in the Ascomycota (with the notable exception of the Saccharomycotina) (Figure 2). Similarity of DmtA to its homologues in *Aspergillus oryzae* (61.5% identity),
Aspergillus fumigatus (69.4% identity), and Aspergillus terreus (59.9% identity) extends across their entire predicted polypeptides. Among characterized proteins, DmtA is most similar to N. crassa RID (41.4% identity) [20] and A. immersus Masc1 (43.3% identity) [21], the putative DMTs involved in RIP and MIP, respectively. Conserved regions are restricted predominantly to the ~300 aa catalytic DMT domain. Like other putative DMTs in the Masc1/RID family, DmtA has conserved DMT domain motifs arranged as in most eukaryotic and prokaryotic DMTs [50,51] and it has a short variable region between motifs VIII and IX (Figure S1). When the sequence of the DmtA polypeptide was scanned against the Conserved Domain Database (CDD) using an E-value threshold of 1.0, we found a Bromo-Adjacent Homology motif (BAH; residues 136 to 236). BAH motifs are also found in Ascobolus Masc1 and the Coccidioides immitis homologue CIMG_10138.2, but they are absent from most of these putative DMTs. Curiously, we found two RID-like proteins in the genome of the Hymenomycete Coprinopsis cinerea (i.e., CC1G_01237.2 and CC1G_00579.1) but both have interrupted catalytic domains and only one (CC1G_00579.1) has a complete BAH domain (data not shown). Similarly, the genomes of Sclerotinia sclerotiorum (SS1G_05055.1 and SS1G_00377.1) and Botryotinia fuckeliana (BC1G_09864.1 and BC1G_00275.1) encode two putative RID homologues. Like Ascobolus Masc1, DmtA homologues from all Aspergillus species, Coccidioides, Uncinocarpus, Botryotinia, and Sclerotinia lack a carboxy-terminal domain after the catalytic domain. This C-terminal region is most prominent in Neurospora species (Freitag et al., 2002), but shorter versions are also found in Chaetomium, Gibberella, Nectria, and Magnaporthe.

The dmtA/tmdA locus is not essential

To investigate the possible biological function of the dmtA/tmdA region, we deleted a 775 bp fragment within the predicted coding region of dmtA and replaced it with the argB gene. The deleted DNA fragment contained most of the predicted catalytic domain of DmtA and included a part of the tmdA transcript. To do this, we transformed A. nidulans strain FGSC A851 with linearized pDLAM002, and selected for growth on medium without arginine (Figure 3A), thereby generating strain DLAN2 (dmtA−/tmdA−). We also evaluated the biological relevance of tmdA by deleting a 645 bp fragment corresponding to its predicted promoter, and by replacing it with the argB marker from pDLAM018 (Figure 3B) in strain FGSC A851 to generate strain DLAN3 (dmtA+/tmdA−). Although we had to search through more than 30 strains to find the desired replacements in each case, the fact that we were able to build the strains implies that the dmtA/tmdA locus does not encode essential gene products. Morphological phenotypes were not detected on these mutants during vegetative growth.

Transcripts originating from the dmtA/tmdA locus are scarce and constitutively produced

No dmtA or tmdA transcripts were detectable by Northern blot hybridizations using 50 μg of total RNA extracted from vegetative mycelium and self-fertilized fruiting bodies of a wild type strain (data not shown). However, both dmtA and tmdA transcripts were detectable by Strand-Specific Reverse Transcribed-PCR in mRNA from wild-type vegetative cells (Figure 4). Results from these experiments suggested that the dmtA/tmdA region is constitutively transcribed on both strands during asexual and sexual development (Figure 4 and data not shown). The anti-sense tmdA transcripts appear slightly more abundant than those of dmtA (Figure 4), which may be why all ESTs sequenced from this region correspond to tmdA. As expected, neither dmtA nor tmdA transcripts were detected in the dmtA+/tmdA− DLAN2 strain (Figure 4).

The existence of non-coding anti-sense transcripts in the dmtA/tmdA region suggested the possibility of functional interactions between the sense and anti-sense transcripts. In such a model, antisense transcripts may serve to regulate the level of DmtA protein by forming double-stranded RNA (dsRNA), which then would be expected to serve as a target for ribonucleases like Dicer [52,53]. If this were the case, our inability to readily detect transcripts by standard Northern blot analysis might reflect rapid degradation, e.g., into small-interfering RNAs (i.e., siRNAs). However, we found that disruption of the promoter controlling the production of tmdA transcript in strain DLAN3 (dmtA+/tmdA−) failed to increase the level of dmtA transcripts significantly, arguing...
against this scenario (Figure 4 and data not shown). We also sought to perturb potential dsRNA formation by increasing \( \text{dmtA} \) transcript levels in \( \text{trans} \). For this, we over-expressed \( \text{dmtA} \) by constructing a transcriptional fusion between the inducible \( \text{niiA} \) (nitrite reductase) promoter [39], and the \( \text{dmtA} \) coding region. The resulting plasmid (pDLAM044) was linearized and used to transform strain FGSC A851. Transformants were selected on minimal medium supplemented with \( \text{p}-\text{aminobenzoic acid} \) and strains with a single ectopic copy of the \( \text{niiA}(\text{p})::\text{dmtA}^+::\text{argB}^+ \) fusion construct were identified by Southern hybridization. Over-expression of \( \text{dmtA}^+ \) in DLAN4 (\( \text{niiA}(\text{p})::\text{dmtA}^+ \)) strain was confirmed by Northern analysis and did not result in RNA degradation or other noticeable phenotypes (data not shown).

\( \text{dmtA} \) is dispensable for asexual development but essential for sexual development

We tested if \( \text{dmtA}^-/\text{tmdA}^-/ \) (DLAN2), \( \text{dmtA}^+/-\text{tmdA}^+/- \) (DLAN3) and \( \text{nicI}(\text{p})::\text{dmtA}^+/-\text{tmdA}^+/- \) (DLAN4) strains were defective in the development and formation of asexual reproductive structures or conidiophores, and if the resulting asexual spores (i.e., conidia) could germinate normally. In all mutants, we found that asexual development, spore formation and spore germination were indistinguishable from those of wild-type strains.

To determine if \( \text{dmtA} \) or \( \text{tmdA} \) are required for sexual development, we self-fertilized strains DLAN2 and DLAN3 and compared the crosses to those of wild-type strains. Interestingly, a \( \text{dmtA}^-/\text{tmdA}^- \) mutant (DLAN2) formed exclusively immature fruiting bodies without ascospores (Figures 5B and 5E) whereas control crosses of wild-type strains produced normal fruiting bodies with abundant sexual spores (Figures 5A and 5D). In contrast, the sexual development of the \( \text{dmtA}^+/-\text{tmdA}^- \) (DLAN3) mutant was indistinguishable from that of the wild type (Figures 5C and 5F).

To ensure that the phenotype observed in \( \text{dmtA}^-/\text{tmdA}^- \) disruption strains was not caused by an unlinked mutation introduced during transformation, both DLAN2 and DLAN3 strains were crossed to DLAN5 (Table 2). Ascospores from these backcrosses were germinated and a total of 24 recombinants (12 per cross) carrying either the \( \text{dmtA}^-/\text{tmdA}^- \) or the \( \text{dmtA}^+/\text{tmdA}^- \) allele, were selected and tested for their ability to undergo homothallic sexual development. Results from this experiment were consistent with our previous observation. All 12 \( \text{dmtA}^-/\text{tmdA}^- \) progeny (from the \( \text{dmtA}^-/\text{tmdA}^- \times \text{dmtA}^-/\text{tmdA}^- \) cross) were
DMTA Is Required for Sex

Figure 5. Sexual development is abolished in dmtA/tmdA mutants but unaffected in dmtA/tmdA mutants and niiA(p)::dmtA transformants. Panels A, B, C, G, H and I show representative cleistothecia from self-fertilized wild type dmtA/tmdA (FGSC A851), the dmtA/tmdA mutant (DLAN2), the dmtA/tmdA mutant (DLAN3), the DmtA overexpressing niiA(p)::dmtA[2334–4547] transformant (DLAN4), and the back-crossed dmtA/tmdA (DLAN6) and dmtA/tmdA mutants (DLAN7), respectively. Cleistothecia were obtained as described in Materials and Methods, cleaned, crushed and photographed using a Zeiss light microscope at 400X magnification. The area highlighted by the rectangles in Panels A, B, C, G, H and I was enlarged six-times and presented in Panels D, E, F, J, K, and L, respectively.

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sterile (DLAN6. Figures 5H and 5K—these figures show the presence of maternal tissue exclusively), whereas the 12 dmtA/tmdA progeny (from the dmtA/tmdA x dmtA/tmdA cross) were fertile (DLAN7. Figures 5I and 5L). Another control cross (FGSC A851 X PW1, Table 2), yielded four recombinants with the same genotype as FGSC A851, and as expected, all these strains were fertile and yielded a normal number of ascospores (data not shown). We conclude that strains lacking dmtA transcript are unable to complete normal sexual development.

We also observed that when self-fertilized and tested for sexual development, cultures of strains carrying the niiA(p)::dmtA; dmtA/tmdA (DLAN4) alleles formed normal fruiting bodies with the expected abundance of fully developed and viable ascospores (Figures 5G and 5J). Together, these results suggest that the absence of dmtA transcript results in a pronounced defect in early sexual development.

Search for DNA methylation in dmtA/tmdA mutants

To test the possible effects of dmtA and tmdA mutations, or overexpression of dmtA, on DNA methylation, we performed two types of experiments. We analyzed genomic DNA of wild type and mutant strains for methylation by traditional Southern hybridization as well as by immunoblotting with a sensitive monoclonal antibody against 5-methylcytosine (5MeC; methods described in [54]. We tested DNA from both vegetative and sexual tissues (cleistothecia) of DLAN2 (dmtA/+/tmdA+), DLAN3 (dmtA+/tmdA+), FGSC 4 (dmtA+/tmdA+), and vegetative tissues of FGSC A851 (dmtA+/tmdA+) and DLAN4 (niiA(p)::dmtA+; dmtA+/tmdA+). DNA from vegetative tissue of a N. crassa wild type (dim-2) and of a dim-2 mutant (which lacks all detectable DNA methylation; [12], served as controls. For Southern hybridizations, DNAs were digested with Sau3AI (S) or DpnII (D), which differ in sensitivity to 5MeC (S is inhibited by 5MeC whereas D is not), and probed with rDNA, which is typically methylated in organisms that sport 5MeC (Figure S2). We were unable to detect DNA methylation in vegetative or sexual tissue from Aspergillus wild type strains by using the Neurospora rDNA repeat and several known methylated retrotransposon relics from Neurospora (Figure S2 and data not shown). We next used the Aspergillus rDNA intragenic spacer region as a probe but did not find DNA methylation (data not shown). No reproducible differences in band patterns between any of the dmtA/tmdA mutants and wild type Aspergillus were found. Tests with the sensitive antibody to 5MeC showed no DNA methylation in any of the Aspergillus strains tested, as with negative control genomic DNA from S. cerevisiae and the Neurospora dim-2 mutant (data not shown). Quantitative differences in DNA methylation levels of four different Neurospora dim mutants previously characterized by Southern hybridizations were confirmed with the antibody assay, suggesting that small changes in DNA methylation levels are detectable by this method (data not shown).

We also tested the effect of 5-azacytidine (5-AC) on strains DLAN2 (dmtA/+/tmdA−), DLAN3 (dmtA+/tmdA−), FGSC 4 (dmtA+/-tmdA+), and FGSC A851 (dmtA+/tmdA+). 5-AC, a cytidine analog is known to cause extensive DNA hypomethylation and its use and mode of action in DNA methylation has been extensively documented [55–57]. In Aspergillus, low concentrations of 5-AC are known to increase the formation of “fluffy” phenotypic variants [47,58], an effect postulated to occur through the heritable modification of a single nuclear gene, fluffy-F1 (FluF1) [58]. The frequency of non-conidial (i.e., fluffy) strains obtained in
our experiments although lower from what was previously reported [47], was nevertheless similar among the different strains tested, thus these experiments did not reveal any phenotypic differences between experimental and control strains (data not shown).

Discussion

We report the cloning and characterization of the dmtA gene, predicted to encode a Masc1/RID DMT-like protein from *A. nidulans*, a fungus in which no DNA methylation has been demonstrated. Curiously, we found that dmtA is transcribed on both strands, thus leading to the designation “dmtA/tmdA” for the locus. In eukaryotes, transcription on both top and bottom strands is often associated with gene silencing at either the transcriptional or post-transcriptional level (e.g., via formation of siRNAs). Our results, however, are not consistent with tmdA playing a role in the regulation of dmtA. Instead, we found that the dmtA transcript, but not the tmdA transcript, is essential for normal completion of sexual development.

In *A. nidulans*, sexual reproduction occurs after asexual sporulation has stopped and results in the formation of macroscopic fruiting bodies called cleistothecia. Although the formation of ascogenous tissue is not completely understood in this organism, its development is thought to be similar to ascogenous tissue development in *Neurospora* and *Asco*bolus, where the two nuclei fuse to generate dikaryotic tissue, which then develops further to form a three-celled hook-shaped structure called the crozier [59]. The parental nuclei in the middle cell of the crozier fuse to form a diploid nucleus, which then immediately undergoes meiosis [59]. The four resulting haploid nuclei undergo a second mitotic division that results in the formation of eight bright red mature ascospores in an ascus. During ascospore maturation in *A. nidulans*, the nuclei undergo a second mitotic division that results in the formation of eight bright red mature binucleate ascospores. It is noteworthy that neither of these early structures can be observed in *dmtA*/*tmdA* mutants. In *Asco*bolus, crosses homozygous for *masc1* are blocked in sexual development before crozier formation [21]. No such defect was observed in *Neurospora* crosses homozygous for *rid* [20]. The *dmtA/tmdA* deletion phenotype observed in Aspergillus resembles the one seen in *Asco*bolus crosses homozygous for *masc1*-cleistothecia are devoid of internal ascogenous tissue. That development of croizers and viable ascospores was never observed in our *dmtA* mutant suggests that DmtA has an important role in sexual development, as previously suggested for Masc1 [21].

It is interesting to consider why a gene known to be involved in RIP in *Neurospora* would be quite conserved and functional in an organism apparently devoid of active RIP and DNA methylation. All Aspergillus species examined to date (*A. nidulans*, *A. fumigatus*, *A. terreus* and *A. oryzae*) have DmtA homologues. DmtA homologues are not restricted to the Eurotiales (e.g., *Aspergillus*), Sordariales (e.g., *Neurospora*), or Pezizales (e.g., *Asco*bolus), but are also present in the Leotiales (e.g., *Sclerotinia*). Among the Ascomycota, they are notably absent from the Saccharomycotina, which also lack components of RNA silencing machinery [61,62].

The predicted structures of DmtA homologues, although conserved, are not identical. These proteins can be grouped by the presence of a conserved BAH domain upstream of the “catalytic” domain. Another feature that distinguishes classes of DmtA homologues is the carboxy-terminal domain. Some DmtA homologues have centrally located catalytic domains (e.g., RID from *N. crassa*, *N. tetrasperma*, *N. intermedia*, and *Podospora anserina*), a second group has a carboxy-terminal located catalytic domain (e.g., DmtA from *A. nidulans*, *A. fumigatus*, *A. oryzae* and *A. terreus*), the two RID homologues each from *Botryotinia fuckeliana* and *Sclerotinia sclerotorum*, Masc1 from *A. immersus*, and RID from *C. immitis* and *Uncinocarpus resinae*). In the remaining members, the carboxy-terminally located peptides have varied lengths (e.g., RID from *Chaetomium globosum*, *Gibberella zeae*, *Nectria hamatococca* and *Magnaporthe grisea*). Based on these comparisons it is tempting to speculate that the C-terminal “tail” of *Neurospora* and *Podospora* RID homologues might be involved in determining RIP efficiency.

Why would the *dmtA/tmdA* locus be transcribed on both strands? In general, our understanding of the interplay of sense/anti-sense transcription is quite widespread and, contrary to an early suggestion, is not restricted to imprinted genes [63]. The ratio of sense/anti-sense transcripts in mouse cells fluctuates markedly among different tissues consistent with the hypothesis that anti-sense transcription serves as a gene regulatory mechanism during development. Interestingly, murine anti-sense transcripts tend to be poly(A)-negative and nuclear localized, similarly to what has been found among randomly selected sense/anti-sense pairs from *Arabidopsis thaliana*, which are also poly(A)-negative and nuclear localized [64]. Perhaps the presence of these sense/anti-sense dsRNA pairs and/or their polyadenylation status determines their nuclear localization in a developmentally regulated manner.

Initial evidence for the existence of sense and anti-sense dsRNA in fungi came from studies aimed at isolating meiosis-specific genes in *Schizosaccharomyces pombe* [65]. Among several meiotic expression upregulated (meu) cDNAs characterized, five lacked clear ORFs and were postulated to represent non-coding RNA. In a more recent study, the presence of long ORFs on both strands of regions of several, distantly-related fungal genomes was taken as an indication for the presence of anti-sense transcripts [66]. The authors of this study noted that, curiously, the majority of the postulated transcript pairs have no homolog in any other characterized species, similar to the situation in mouse. They also proposed that the genes involved in sense/anti-sense-relationships code for proteins that are preferentially localized to the nucleus, as suggested by the Gene Ontology terms used to annotate them. The *IME4* gene of *S. cerevisiae* exemplifies the importance of sense-anti-sense transcription in fungi. The *IME4* anti-sense transcript causes transcriptional interference, a regulatory mechanism that controls entry into meiosis [67]. Our observation that the *dmtA/tmdA* locus is actively transcribed in both directions constitutes the first demonstration of the presence of a sense/anti-sense-pair that, analogous to the *IME4* locus in yeast, controls the early stages of sexual development in *A. nidulans*.

Finally, we did not detect DNA methylation in *A. nidulans*. This is consistent with previous studies that failed to reveal DNA methylation or MIP in this organism [47,58,68]. Similarly, RIP has not been detected in *A. nidulans*, despite numerous studies in which RIP, if present, should have been observable even at a low level, as was reported for *Leptosphaeria maculans* [69,70] and *P. anserina* [71–73]. Nevertheless, it seems possible that DmtA proteins are responsible for vestiges of RIP found in the *Aspergillus* genome and perhaps for the low RIP activity reported for some other *Aspergillus* species [74–76]. One possibility is that in *Aspergillus de novo* DNA methylation by DmtA only occurs transiently during the sexual phase, a stage where low levels of DNA methylation are difficult to detect. If true, this methylation might not be maintained and might only rarely result in RIP. Future studies on the expression of DmtA or Masc1 in *Neurospora* may provide clues to what makes a fungus competent for efficient MIP or RIP.
Supporting Information

Figure S1
Found at: doi:10.1371/journal.pone.0002531.s001 (0.57 MB PDF)

Figure S2
Found at: doi:10.1371/journal.pone.0002531.s002 (0.59 MB PDF)

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Conceived and designed the experiments: ES RA DL MF. Performed the experiments: RA DL MF. Analyzed the data: ES RA DL MF. Contributed reagents/materials/analysis tools: ES RA DL MF. Wrote the paper: ES RA MF.
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