The Putative C2H2 Transcription Factor MtfA Is a Novel Regulator of Secondary Metabolism and Morphogenesis in *Aspergillus nidulans*

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**Abstract**

Secondary metabolism in the model fungus *Aspergillus nidulans* is controlled by the conserved global regulator VeA, which also governs morphological differentiation. Among the secondary metabolites regulated by VeA is the mycotoxin sterigmatocystin (ST). The presence of VeA is necessary for the biosynthesis of this carcinogenic compound. We identified a revertant mutant able to synthesize ST intermediates in the absence of VeA. The point mutation occurred at the coding region of a gene encoding a novel putative C2H2 zinc finger domain transcription factor that we denominated *mtfA*. The *A. nidulans mtfA* gene product localizes at nuclei independently of the illumination regime. Deletion of the *mtfA* gene restores mycotoxin biosynthesis in the absence of veA, but drastically reduced mycotoxin production when *mtfA* gene expression was altered, by deletion or overexpression, in *A. nidulans* strains with a veA wild-type allele. Our study revealed that *mtfA* regulates ST production by affecting the expression of the specific ST gene cluster activator *aflR*. Importantly, *mtfA* is also a regulator of other secondary metabolism gene clusters, such as genes responsible for the synthesis of terrequinone and penicillin. As in the case of ST, deletion or overexpression of *mtfA* was also detrimental for the expression of terrequinone genes. Deletion of *mtfA* also decreased the expression of the genes in the penicillin gene cluster, reducing penicillin production. However, in this case, over-expression of *mtfA* enhanced the transcription of penicillin genes, increasing penicillin production more than 5 fold with respect to the control. Importantly, in addition to its effect on secondary metabolism, *mtfA* also affects asexual and sexual development in *A. nidulans*. Deletion of *mtfA* results in a reduction of conidiation and sexual stage. We found *mtfA* putative orthologs conserved in other fungal species.

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**Introduction**

Fungal species produce numerous secondary metabolites [1,2,3], including compounds with detrimental effects, such as mycotoxins [4], capable of causing disease and death in humans and other animals [4,5]. *Aspergillus nidulans*, a model filamentous fungus studied for more than fifty years, produces the mycotoxin sterigmatocystin (ST). This mycotoxin, ST, and the well-known carcinogenic compounds called aflatoxins (AF), produced by related species such as *A. flavus*, *A. parasiticus*, and *A. nomius* [6], are both synthesized through a conserved metabolic pathway [7,8,9] where ST is the penultimate precursor. The genes responsible for ST/AF production are clustered. Within these clusters, the regulatory gene *aflR* encodes a transcription factor that acts as a specific cluster activator [10,11,12].

The range of secondary metabolites produced by *A. nidulans* also includes bioactive compounds with demonstrated beneficial effects and applications for medical treatments, including antibiotics, such as the beta-lactam penicillin (PN) [13,14], or anti-tumoral metabolites such as terrequinone [15,16], with potential direct application in the medical field. In both cases the genes involved in the synthesis of these compounds are also found clustered [16,17].

In fungi, secondary metabolism is often found to be governed by genetic mechanisms that also control asexual and sexual development [18]. One of these principal common regulatory links is the global regulatory gene *veA*, first described to be a developmental regulator in *A. nidulans* [19,20]. In 2003 we described for the first time the connection between *veA* and the synthesis of diverse fungal secondary metabolites, including ST [21]. Absence of the *veA* gene in *A. nidulans* prevents *aflR* expression and concomitant ST biosynthesis. A similar effect was also observed in *Aspergillus flavus* and *Aspergillus parasiticus* *veA* deletion mutants, that lost the capacity to produce AFs [22,23,24]. Furthermore, *veA* also regulates the biosynthesis of other mycotoxins, for example cyclopiazonic acid and aflatem in *Aspergillus flavus* [22]. *veA* is
This effect was also observed in mycotoxigenic F. verticillioides such as P. chrysogenum [21,28] as well as cephalosporin C in Acremonium chrysogenum [29].

VeA has also been found to affect fungal infection of plants and animals. For example, a decrease in virulence was observed in deletion veA mutants of A. flavus when infecting plant tissue [24]. This effect was also observed in mycotoxigenic Fusarium species, such as F. verticillioides [25], F. graminearum [26] and F. fujikuroi [27]. In the case of animal infections, deletion of the veA homolog in Histoplasma capsulatum also leads to a reduction in virulence in a murine model [30], although in Aspergillus fumigatus veA is dispensable for virulence in the neutropenic mouse infection model [31].

Most of the studies to elucidate the veA regulatory mechanism of action have been carried out using the model fungus A. nidulans. It is known that the KapA- importin transports the VeA protein to the nucleus, and that this transport is promoted by darkness [32,33]. In the nucleus, VeA interacts with light-responsive proteins that also modulate mycotoxin production and fungal development, such as the red phytochrome-like protein FphA, which interacts with the blue sensing proteins LrcA-LrcB [34,35]. VeA also sustains other nuclear protein interactions with VelB and LaeA [36,37]. LaeA, a chromatin modifying protein, is also required for the synthesis of ST and other secondary metabolites [38,39]. Absence of VelB, another protein of the velvet family [37], decreases and delays ST biosynthesis, indicating a positive role in ST biosynthesis [36].

To identify novel veA-dependent genetic elements involved in the regulation of ST biosynthesis in the model system A. nidulans, we performed a mutagenesis in a deletion veA strain to generate revertant mutants that regained the capacity to produce toxin [40]. Several revertant mutants (RM) were obtained. In the present study we characterized one of these selected revertants, RM7. This revertant mutant presented a point mutation in a gene that we denominated mtfA (master transcription factor A) encoding a novel putative C2H2 zinc finger domain type transcription factor. We show that the mtfA effect on ST production is veA-dependent. Additionally, mtfA regulates the expression of other secondary metabolite gene clusters, such as those of terrequinone and PN. Furthermore, mtfA is also important for normal sexual and asexual development in A. nidulans.

### Table 1. Fungal strains used in the study.

| Strain name | Pertinent genotype | Source |
|-------------|--------------------|--------|
| FGSC4       | Wild type (veA+)   | FGSC   |
| RDAE206     | yA2, paboA1, pyrG89; argB2, ΔstcE:argB, ΔveA:argB | FGSC   |
| RDAEp206    | yA2, ΔstcE:argB, ΔveA:argB | [40]   |
| RAV1        | yA2, paboA1, pyrG89; argB2, ΔstcE:argB, veA1 | [40]   |
| RAV1p       | yA2; WA3; ΔstcE:argB; veA1 | [40]   |
| RAV2        | yA2; WA3; argB2, ΔstcE:argB; pyroA4; veA1 | [40]   |
| RM7         | yA2, paboA1, pyrG89; argB2, ΔstcE:argB, ΔveA :argB, mtfA- | This study |
| RM7p        | yA2, ΔstcE:argB, ΔveA :argB, mtfA-- | This study |
| RM7-R2      | yA2, pyrG89; WA3; argB2, ΔstcE:argB, mtfA-- | This study |
| RM7p-R2     | yA2; WA3; ΔstcE:argB, mtfA-- | This study |
| RM7-R2-com  | yA2, pyrG89; WA3; argB2, ΔstcE:argB, mtfA-- | This study |
| RMP1.49     | pyrG89; argB2, ΔnkuA:argB; pyroA4; veA+ | [71]   |
| TRVS0.1     | argB2, ΔnkuA:argB; pyroA4; veA+ | This study |
| TRVS0.2     | argB2, ΔnkuA:argB; veA+ | This study |
| TRVΔmtfA    | pyrG89; argB2, ΔnkuA:argB; ΔmtfA:pyrG4*::fum; pyroA4; veA+ | This study |
| TRVΔmtfAα   | ΔnkuA:argB; ΔmtfA:pyrG4*::fum; veA+ | This study |
| TRVΔmtfA-C   | pyrG89; argB2, ΔnkuA:argB; ΔmtfA:pyrG4*::fum; pyroA4;mtfA; pyroA4; veA+ | This study |
| TRV60       | pyrG89; argB2, ΔnkuA:argB, alcA(p);mtfA:pyr4; pyroA4; veA+ | This study |
| TDAEΔmtfA   | paboA1, pyrG89; ΔmtfA:pyrG4*::fum; ΔstcE:argB, ΔveA :argB | This study |
| TDAEΔmtfAα  | pyrG89; ΔmtfA:pyrG4*::fum, ΔstcE:argB, ΔveA :argB | This study |
| RJW41.1     | ΔnkuA; veA+ | [36]   |
| RDAI2.3     | veA1 | [39]   |
| RJW46.4     | ΔnkuA; veA1 | [39]   |
| RSD10.1     | pyrG89; WA3; argB2, ΔnkuA:argB; ΔmtfA:pyrG4*::fum; ΔnkuA:mehtG; veA1 | This study |
| RSD11.2     | pyrG89; WA3; argB2, ΔnkuA:argB; ΔmtfA:pyrG4*::fum; ΔnkuA:mehtG; veA+ | This study |
| TSD12.1     | pyrG89; ΔnkuA:argB; mtfA:gfp:pyrG4*::fum; pyroA4 | This study |

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Materials and Methods

Fungal Strains and Growth Conditions

Fungal strains used in this study are listed in Table 1. Media used include glucose minimal media (GMM) [41], YGT (0.5% yeast extract, 2% dextrose, trace elements prepared as described [41], and oat meal media (OMM) (1% oat meal). Supplements for auxotrophic markers were added as required [41]. Glucose was substituted with threonine (100 mM) in threonine minimal medium (TMM) for induction of alcA promoter. Solid medium was prepared by adding 10 g/liter agar. Strains were stored as 30% glycerol stocks at −80°C.

Genetic Techniques

Meiotic recombination between A. nidulans strains was carried out as previously described [42]. Progeny from the cross between the RM7 mutant [40] and RAV2 (yA2, wA3, argB2, ΔstcE::argB, pyrA4) were analyzed for the presence or absence of veA by PCR. Colony morphology, as well as norsolorinic acid (NOR) production, were also studied. The progeny of this cross showed four phenotypic groups: 1. ΔveA, ΔstcE, X- (RM7 parental type); 2. ΔstcE (RAV2 parental type); 3. recombinant ΔveA, ΔstcE (RM7-R1) and recombinant ΔstcE, X- (RM7-R2). Dominance test was carried out by forming diploids with RM7-R2 and RAV1 strain.

Table 2. Primers used in this study.

| Name        | Sequence (5’ → 3’)                          |
|-------------|---------------------------------------------|
| RM7-F1      | TACGCGGATTCACCTCACTGGGCG                    |
| RM7-R1      | TAACCTACGCGAAGCAGCGCGCG                    |
| RM7com1     | AAAACCGGGGAGTCAGTAGAGGAGTGT               |
| RM7com2     | AAAAAGTACCGCGTAGACTGATTGCTTCTC            |
| RM7-OE1     | AAAAAGTACCATGATGTGAGACCTCCCATGTCC         |
| RM7-OE2     | AAAAATTATAATTACTACACCTCGGAGCAGCGCC       |
| actin-F     | ATGGAAGAGGGAGATTGCCCTCCTCACTCGGACAATGTTC |
| actin-R     | CAATGGAGGGAGAAGACGGCGAAGG                 |
| aflrF       | GAGCCCCCAGCGATAGC                         |
| aflrR       | CGGGGTGTGTGCTGTGCC                        |
| stcU-F      | TTATCTAAAGGCCCCCCCCATCAA                 |
| stcU-R      | ATGCGCTCTCTCCGGGATTTGACCAGTC             |
| nsdD-F      | CATCTACAGACGACGACGAGCTCACAGGACTG          |
| nsdD-R      | TTAGGAGCGCCAGACGAGGTGATACACAGGCTCCA       |
| staF        | TCCAGCAAATGGAACGAGTGAACTACGGTGTCTC       |
| staR        | GAAAGGGATGGAGGAGAAGACTGTTCACTCGGAGGATA   |
| brlA-F      | AGCTGCTGCTGGTCTGAGGATGTTGTGGGTTGTTGCC    |
| brlA-R      | CGGAAGAGAAAGGCTCAGCCTCGGCGAGCACAGC       |
| acvA-F      | GAAAAGGACAGAGCCCTAGGAGGGAAGG             |
| acvA_R      | CCGAGCCGACGCCCTCTGCGAAGACAGAC           |
| aat-A       | CCCCTAGCCTCGCCAATGCTGACGCAAA             |
| aat-R       | GCCCTCCGGCCCACTGATGCAAGGAGAC            |
| tdiAF       | GCCCCAATGCTCAGCCGCTCCTCA                   |
| tdiAR       | TCTGCGCTCTGTGGAGAAGGAGGACATC            |
| tdiBF       | CATGACCTACAGCAGCTCCCTCTC                 |
| tdiBR       | GCCCTCTCAAAGTCCGCTG                    |
| mtfAgpF_787 | CCCACCTGATCTCCGCACTC                      |
| mtfAgpR_788 | CACCGATCGGAGCGATCC                |
| mtfA3_F_789 | CCAATGTTGCTCAATCCTCGTGTCTC              |
| mtfA3_R_790 | TGGAGTCTGCTGGCGAGGCCCTGAG             |
| mtfAlinkerF_791 | AGGCCGTGCGCTGGTAGTTGAGGAGGCGGTCCTCA       |
| mtfAlinkerR_792 | AGGCCGTGCGCTGGTAGTTGAGGAGGCGGTCCTCA       |
| aflR06038   | ATGGAGGCCCCAGCGATCAGCAGG                |
| aflR06039   | TTAGGAGTAGGGCTGCTGCTGCTGCTGCTCAAC       |
| mtfA13015   | GCCCTCACCCTCCTAGGCGGAAATG              |
| mtfA13016   | GGTGCGGAGCGGCTGCTGAGG                  |

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Identification of the Revertant Mutation in RM7

To find the mutation in RM7, A. nidulans genomic library pRG3-AMA1-NOT1 was utilized to transform the RM7-R2 (ΔstcE, X−) strain. Plasmid DNA was rescued from fungal transformants presenting wild-type phenotype. Both end regions of the DNA inserts in the isolated plasmids were sequenced and the complete insert sequences were found in the A. nidulans genome database [http://www.aspgd.org] by BLAST analysis. The exact location of the mutation in RM7 was identified by sequencing of the PCR product amplified from the same locus in RM7.

Sequence Search and Alignment

The deduced protein sequence of MtfA (AN87412.2) was compared against databases from different fungal genera, using the BLAST (blastp) tool provided by National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/]. The gene entry with the highest percentage of identity and the lowest e-value for each of the species was selected (Table S1). Pairwise sequence alignment of the proteins was performed using the EMBOSS Needle tool [http://www.ebi.ac.uk/Tools/psa/emboss_needle/] from EMBL-EBI (European Molecular Biology Laboratory’s European Bioinformatics Institute). Percentage of similarities and percentage of identities were tabulated for each of the alignments (Table S1). Multiple sequence alignment was performed using MUSCLE v3.8.31 [43]. The alignment was used to build a Hidden Markov model (HMM), followed by realignment of sequences against the generated HMM, using the hmmbuild and hmmalign tools in HMMER v3.0b2 [http://hmmer.org/]. A maximum likelihood phylogeny reconstruction method implemented in the software PhyML v3.0 [44,45], whose workflow is available at iPlant collaborative [http://www.iplantcollaborative.org/] was used for tree construction with default settings. The resulting tree was viewed using FigTree v1.4.0 [http://tree.bio.ed.ac.uk/software/figtree/]. Midpoint rooting [46] of the tree was chosen in order to minimize the large distances from the root to any leaf. The numbers on the branches indicate the approximate likelihood branch support values in percentages [47].

Generation of the mtfA Deletion Strain

The entire mtfA coding region was replaced in RDAE206 and RJMP1.49 strains (Table 1). The DNA cassette used to delete mtfA by gene replacement with the pyrG marker was obtained from FGSC [http://www.fgsc.net]. Polyethylene glycol-mediated transformation of RDAE206 and RJMP1.49 protoplasts was carried out as described previously [48]. Transformants were selected on appropriate selection medium without uridine or uracil and confirmed by Southern blot analysis as previously described [49]. The deletion strains were designated as TDAEΔmtfA and TRVΔmtfA respectively.

A complementation strain was also obtained by transforming ΔmtfA (TRVΔmtfA) strain with the mtfA wild-type allele. The complementation vector was generated as follows: A DNA fragment contained the entire mtfA coding region and 5′ and 3′ UTRs was first amplified with primers RM7comI and RM7com2 (Table 2) from FGSC4 A. nidulans genomic DNA. Then the PCR product was digested with SacI and KpnI and cloned into pSM3 vector, containing the pyrG transformation marker, previously digested with the same enzymes, resulting in the plasmid pSM3RM1. This vector was transformed into ΔmtfA protoplasts and the transformants were selected on appropriate selection medium without pyridoxine. Complementation was confirmed by PCR and Southern blot analysis. The complemented strain was
designated as \( \Delta \text{mtfA} \)-com. Strains that were isogenic with respect to the auxotrophic markers were generated and used in this study.

**Generation of \( \text{mtfA} \) Over-expression Strain**

To generate the \( \text{mtfA} \) over-expression strain, the entire \( \text{mtfA} \) coding region was first amplified using the RM7-OE1 and RM7-OE2 primers (Table 2). The PCR product was then digested with \( KpnI \) and \( PstI \) and ligated into pmacro plasmid, containing the \( A. \text{nidulans} \) alcA promoter, \( \text{trpC} \) terminator and \( \text{pyrG} \) marker, resulting in the plasmid pMacroMtfAOE. The pMacroMtfAOE vector was transformed into RJMP1.49 and transformants were selected on appropriate selection medium without uridine and uracil, and confirmed by PCR using RM7-OE1 and RM7-OE2 primers.

[Figure 2. RM7 mutant presents a single gene mutation at locus AN8741.2. A) Diagram showing the genomic insert present in the complementation vector from library pRG3-AMA1-NOT1. The insert contains two ORFs corresponding to AN8741.2 and adjacent AN8740.2. The coding region at AN8741.2 locus encodes a putative \( C_2H_2 \) zinc finger domain transcription factor. The revertant mutation in RM7 occurred at AN8741.2, designated as \( \text{mtfA} \) gene. B) Amino acid alignment of \( A. \text{nidulans} \) MtfA (Ani) and putative orthologs in \( A. \text{terreus} \) (Ate), \( A. \text{flavus} \) (Afl), \( A. \text{clavatus} \) (Acl) and \( A. \text{fumigatus} \) (Afu). ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.htm) and boxshade (http://www.ch.embnet.org/software/BOX_form.html) multiple sequence alignment software programs were utilized in this analysis. The mutation occurred at the codon corresponding to the first methionine. The two conserved zinc finger domains are indicated in A) and B).](http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0074122.g002)
Figure 3. Effects of \textit{mtfA} deletion on ST production in \textit{A. nidulans} strains with a \textit{veA}+ allele. A) TLC analysis showing ST production in GMM cultures. Wild type (WT) veA+ control (TRV50.2), Δ\textit{mtfA} (TRVpΔ\textit{mtfA}) and Δ\textit{mtfA}-com complementation strain (TRVΔ\textit{mtfA}-com) were spread-inoculated with 5 mL of top agar containing 10^6 conidia mL^21 and incubated at 37°C in the dark or in the light for 48 h and 72 h. ST was extracted and analyzed by TLC as described in the Material and Methods section. White arrows indicate unknown compounds whose synthesis is also affected by the presence or absence of \textit{mtfA}. B) Effect of the \textit{mtfA} deletion on \textit{aflR} and \textit{stcU} expression. Wild type (WT) veA+ control (TRV50.2), Δ\textit{mtfA} (TRVpΔ\textit{mtfA}) and Δ\textit{mtfA}-com complementation strain (TRVΔ\textit{mtfA}-com) were inoculated in liquid GMM. Mycelia were collected 24 h and 48 h after inoculation. Cultures were grown in a shaker incubator at 37°C at 250 rpm. Expression of \textit{aflR} and \textit{stcU} was analyzed by Northern blot. 18S rRNA serves as loading control. Asterisk indicates not detected. C) TLC showing accumulation of ST in the cultures described in (B). Densitometries were carried out with the Scion Image Beta 4.03 software.

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strains were grown in GMM liquid shaken cultures (10^6 spores/mL were collected and extracted with chloroform. Alternatively, dark. Three cores (16 mm diameter) from each replicate plate were then spread onto inducing medium TMM. Culture supernatants were also extracted with chloroform. Samples were collected for RNA analysis 24 and 48 hrs after shift. Culture supernatants were collected for toxin analysis and mycelia were resuspended in 200 µL of chloroform. Samples were fractionated by silica gel thin-layer chromatography (TLC) using benzene and glacial acetic acid (95:5, v/v) as solvent system for ST analysis and chloroform:acetone:n-hexane (85:15:20) for NOR analysis. Aluminiun chloride (15% in ethanol) was sprayed on the plates, followed by glacial acetic acid [95:5 (v/v)] as solvent system for ST analysis. Data were visualized under UV light (375-nm).

RNA Analysis
Culture plates containing 25 mL of solid GMM or OMM with appropriate supplements were top-agar inoculated with approximately 5×10^6 spores/mL. The cultures were incubated in the dark. Three cores (16 mm diameter) from each replicate plate were collected and extracted with chloroform. Alternatively, strains were grown in GMM liquid shaken cultures (10^6 spores/mL) and incubated at 37°C. Twenty-four h and 48 h old culture supernatants were analyzed for ST and mycelia were collected for RNA analysis. For analysis of mycoxin production in over-expression mtfA and control cultures, GMM was inoculated with conidia (10^6 conidia/mL) from the mtfA over-expression strain (TRV60) or its isogenic control (TRV50.1), and incubated for 16 h at 37°C and 250 rpm. Then, equal amounts of mycelium were transferred and spread onto TMM agar medium. The cultures were further grown for 48 h and 72 h. Mycelial samples were collected at 0 h (shift time), and 24 and 48 hrs of incubation after shift onto TMM. 18S rRNA serves as loading control. B) qRT-PCR expression analysis of mtfA expression. Wild-type isogenic control (WT) veA+ (TRV50.1) and over-expression (OE) mtfA strain (TRV60) were inoculated in GMM liquid medium (10^6 conidia mL⁻¹) and grown for 16 hrs in a shaker incubator at 37°C and 250 rpm. Then, equal amounts of mycelium were transferred and spread onto TMM agar medium. The cultures were further grown for 48 h and 72 h. Mycelial samples were collected at 0 h (shift time), and 24 and 48 hrs of incubation after shift onto TMM. 18S rRNA serves as loading control. B) qRT-PCR expression analysis of mtfA from mycelial samples collected after 24 h and 48 h of incubation after transfer onto TMM agar medium. C) TLC analysis of ST production from cultures described in (A-B). doi:10.1371/journal.pone.0074122.g004

Penicillin Analysis
The PN bioassay analysis was carried out as previously described [50] with some modifications, using Bacillus calidolactis C953 as testing organism. Briefly, strains were inoculated with approximately 10^6 spores mL⁻¹ in 20 mL of seed culture medium, and incubated at 26°C for 24 h at 250 rpm. Mycelia were collected with Miracloth (Calbiochem, USA) and transferred to PN-inducing medium containing lactose, 40 g/L; corn steep liquid (50%), 40 g/L; KH₂PO₄, 7 g/L; and phenoxyacetic acid, 0.5 g/L; pH was adjusted to 6.0 with 10 M KOH. Cyclopentanone (10 mM) was added to induce expression of the scl1 promoter when the mtfA over-expression strain and its isogenic control were used. Twenty mL of PN-inducing medium was inoculated with 1 mL of mycelia suspension (containing equal amounts of mycelium), and mycelial samples were collected at 24 h and 48 h of incubation for RNA analysis. After 96 h, the culture supernatants were collected for PN analysis. The experiment was carried out with three replicates. Three hundred mL of Tryptone-Soy Agar was inoculated with 20 mL of B. calidolactis C953 culture and plated on three 150-mm-diameter Petri dishes. Supernatant aliquots of each culture supernatant were then added to 7-mm-diameter wells. Bacteria were cultured at 35°C for 16 h and inhibition halos were visualized and measured. To confirm that the observed antibacterial activity was due to the presence of PN and not to the presence of other fungal compounds in the supernatant, controls containing commercial penicillinase from Bacillus cereus (Sigma, MO, USA) were also used. A standard curve using various concentrations of PN G (Sigma, MO, USA) was utilized to determine PN concentration in each sample.

Figure 4. Over-expression of mtfA suppresses aflR and stcU expression and ST production. A) Northern blot analysis of aflR and stcU expression. Wild-type isogenic control (WT) veA+ (TRV50.1) and over-expression (OE) mtfA strain (TRV60) were inoculated in GMM liquid medium (10^6 conidia mL⁻¹) and grown for 16 hrs in a shaker incubator at 37°C and 250 rpm. Then, equal amounts of mycelium were transferred and spread onto TMM agar medium. The cultures were further grown for 48 h and 72 h. Mycelial samples were collected at 0 h (shift time), and 24 and 48 hrs of incubation after shift onto TMM. 18S rRNA serves as loading control. B) qRT-PCR expression analysis of mtfA from mycelial samples collected after 24 h and 48 h of incubation after transfer onto TMM agar medium. C) TLC analysis of ST production from cultures described in (A-B). doi:10.1371/journal.pone.0074122.g004

Study of MtfA Subcellular Localization
Aspergillus nidulans RJMP1.49 strain (Table 1) was transformed with mtfA::gfp::pyrG^4::pyrG^4:: as described previously [48]. Primers used in the generation of the fusion PCR product utilized for transformation are listed in Table 2. Plasmid p1439 [32] was used as template for the PCR amplification of the intermediate fragment. Correct integration was confirmed by PCR and

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Southern blot analysis (data not shown). Conidia from the selected transformant (i.e. TSD12.1, Table 1) were inoculated as described previously [32]. Briefly, conidia were allowed to germinate on coverslip submerged in Watch minimal medium [51] in light or dark. After 16 h samples were washed in 1×PBS and stained with DAPI (10 ng/mL) in 50% glycerol and 0.1% Triton X-100. Samples were observed with a Nikon Eclipse E-600 equipped with Nomarski optics and fluorochromes for GFP and UV using a 100× objective. Micrographs were taken using Hamamatsu ORCA-ER high sensitivity monochrome CCD camera using Microsuite 5 imaging software. The exposure time for DIC, DAPI and GFP was 50 ms, 200 ms and 1 s respectively.

Morphological Studies

Plates containing 25 mL of solid GMM with the appropriate supplements were top-agar inoculated with 5 mL of top agar containing 10⁶ spores/mL of A. nidulans strains TRV50.2 control, ΔmtfA or ΔmtfA-com (Table 1). The cultures were incubated in dark or in light at 37°C. Cores were removed from each culture and homogenized in water. Conidia and Hu¨lle cells were counted using a hemacytometer. Identical cores were taken to visualize cleistothecia under a dissecting microscope. To improve visualization of fruiting bodies, the cores were sprayed with 70% ethanol to remove conidiophores.

For radial growth analysis, each strain was point inoculated and incubated under light or dark conditions at 37°C for 6 days, when colony diameter was measured. Experiments were performed with three replicates.

Gene Expression Analysis

Total RNA was extracted from lyophilized mycelia using RNeasy Mini Kit (Qiagen) or Trizol (Invitrogen), following the manufacturer’s instructions. Gene expression levels were evaluated by Northern blots or quantitative reverse transcription-PCR (qRT-PCR) analysis. The templates used for making probes for Northern blots were obtained as follows: ipnA, a 1.1-kb HindIII-EcoRI fragment of pUCHH(458) [52]; aflR, stcU, aatA, acvA, dicA, and dthB probe templates were amplified by PCR from A. nidulans genomic DNA with primers indicated in Table 2.

For qRT-PCR, 2 μg of total RNA was treated with RQ1 RNase-Free DNase (Promega). cDNA was synthesized with Moloney murine leukemia virus (MMLV) reverse transcriptase.
qRT-PCR was performed with the Applied Biosystems 7000 Real-Time PCR System using SYBR green dye for fluorescence detection. The primer pairs used for qRT-PCR are listed in Table 1. The expression data for each gene was normalized to the *A. nidulans* actin gene expression and the relative expression levels were calculated using the $2^{-\Delta\Delta C\text{T}}$ method.

**Results**

**Locus AN8741.2, Mutated in RM7, Encodes a Putative C2H2 Type Transcription Factor**

In our previous study, we generated seven revertant mutants (RMs) capable of restoring normal levels in the production of the orange ST intermediate norsonolinic acid (NOR) in a ΔveA strain lacking the veA gene (RDAE206) [40]. Classical genetics analysis revealed that these RMs belong to different linkage groups (data not shown). In the current study we identify the mutated gene in RM7 that restores toxin production in a deletion veA genetic background (Figure 1). The mutation in RM7 was recessive (data not shown) and the specific affected locus was found by complementation of RM7-R2 with an *A. nidulans* genomic library (pRG3-AMA1-NOT1, [53]). Several positive transformants showing wild-type phenotype were obtained. Sequencing of the rescued plasmids from these fungal transformants and comparison of these sequences with the *A. nidulans* genomic database (http://www.aspgd.org) by BLAST analysis indicated that they contained the same genomic insert including two ORFs, one of them encoding a putative C2H2 finger domain protein, and another encoding an unknown hypothetical protein (Figure 2). In order to determine where the mutation was located in RM7, the corresponding genomic DNA fragment was PCR-amplified. Sequencing of this PCR product revealed that the mutation occurred in a gene encoding the novel putative C2H2 transcription factor, that we designated *mtfA* (*m*aster *t*ranscription *f*actor A). The mutation was a G-T transversion at nucleotide +3 of the *mtfA* coding region, changing the start codon from ATG to ATT (Figure 2A).

**MtfA Orthologs are Present in Other Fungal Species**

The deduced amino acid sequence of *A. nidulans* MtfA revealed significant identity with ortholog proteins from other *Aspergillus* species.

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**Figure 6. Over-expression of mtfA increases penicillin production.** A) Extracts from wild-type (WT) veA+ control (TRV50.1), and over-expression (OE) mtfA strain (TRV60) were analyzed for penicillin content as described in Materials and Methods section. B) qRT-PCR expression analysis of acvA from mycelial samples collected after 24 h and 48 h of incubation in PN inducing medium. C) Northern blot analysis of ipnA and aatA from samples collected after 24 h and 48 h of incubation in PN inducing medium. Densitometries were carried out with the Scion Image Beta 4.03 software.
doi:10.1371/journal.pone.0074122.g006
spp., such as *A. clavatus* (64% identity), *A. terreus* (61%), *A. flavus* (61%), or *A. fumigatus* (59%). Further analysis of other fungal genomic databases indicated that MtfA is also conserved in other fungal genera in Ascomycetes (Table S1, Figure S1 and S2). The C2H2 DNA binding domain is highly conserved among these putative orthologs. A MtfA ortholog was not found in the strict-yeast fungus *Saccharomyces cerevisiae*. Similarly, MtfA putative orthologs were not found in plants or animals. Orthologs from other fungal genera are listed in Table S1. An extensive alignment and phylogenetic tree is shown in Figure S1 and S2. MtfA orthologs were particularly conserved among *Aspergillus* spp. The MtfA tree topology was consistent with established fungal taxonomy. MtfA presents similarity to other *A. nidulans* C2H2 DNA binding domain proteins (Table S2), showing the highest similarity with FlbC (25.3% identity in the full protein comparison and 29% identity when comparing the DNA binding domains).

**mtfA Regulates Mycotoxin Biosynthesis**

To confirm that NOR production in RM7 (ΔstcE, ΔveA) was indeed due to a loss-of-function mutation in *mtfA*, and to assess the effect of this mutation on ST production in a strain with a wild-type *veA* allele (*veA+*), we performed a complete deletion of *mtfA* in RDAE206 (ΔveA) and RJMP1.49 (*veA+*), obtaining TDAEΔmtfA and TRVΔmtfA strains, respectively (Figure S3). Deletion of *mtfA* in these strains was confirmed by Southern blot analysis, using the 5′ UTR as probe template P1 (Figure S3B). This probe revealed a 7.1 kb *Pvu* fragment in the wild-type control and a 6.3 kb *Pvu* fragment in the deletion mutants as expected. Also, hybridization with the transformation marker gene used for gene replacement, *AfpyrG* (specific probe template P2), revealed 6.3 kb and 2.2 kb *Pvu* fragments in *mtfA* deletion mutants, while these bands were absent in the wild-type control (Figure S3B), as predicted.

Similarly to RM7p (ΔstcE, ΔveA, mtfA−) (p, indicates prototrophy), the TDAEΔmtfA (ΔstcE, ΔveA, ΔmtfA) strain shows an increase in NOR production with respect to RDAE206 (ΔstcE, ΔveA, ΔmtfA−), a common *veA* mutant genetic background used in numerous *A. nidulans* research laboratories that still allows ST production. The levels of NOR production by RM7-R2p were similar to those detected in the isogenic control RAV1p (ΔstcE, ΔveA, ΔmtfA) (Figure 1).

To elucidate the role of *mtfA* in mycotoxin biosynthesis in a strain with a *veA* wild-type genetic background (*veA+*) we analyzed ST production in the TRVΔpsmsfA strain and compared it with...
that of the isogenic wild-type control strain and the complementation strain. Interestingly, our results indicated that TRVp
mtfA mutant did not produce ST after 48 h of incubation under both light and dark conditions in the veA wild-type background, whereas the wild type and complementation strain produced clearly detectable levels of ST (Figure 3A). At 72 h only very low levels of ST were detected in the TRVp
mtfA culture under these experimental conditions (Figure 3A). In addition, the TLC analysis indicated that deletion of mtfA also resulted in a delay in the synthesis of two additional unknown compounds in cultures growing in the dark (Figure 3A).

mtfA Controls aflR Expression and Activation of the ST Gene Cluster

Expression of the specific ST regulatory gene aflR, and expression of stcU, gene encoding a ketoreductase that is used as indicator for cluster activation [21,54], were analyzed in liquid shaken cultures of wild type, deletion mtfA and complementation strain at 24 h and 48 h after spore inoculation. Neither aflR nor stcU were expressed in the mtfA deletion mutant, while transcripts for both genes accumulated at the 48 h time point analyzed (Figure 3B). The presence of these transcripts coincided with the presence of ST in the control cultures. Mycotoxin was not detected in the mtfA deletion cultures under the experimental conditions assayed (Figure 3C). Analysis of later time points also showed a notable reduction of ST production as well as a reduction in aflR expression in the ΔmtfA strain with respect to the controls (Figure S4). Over-expression of mtfA (alcA(p)::mtfA, veA+) also prevented the transcription of aflR and stcU as well as ST production under conditions that allowed the control strains to activate the transcription of ST genes and mycotoxin production (Figure 4).

Deletion of mtfA does not Recover Mycotoxin Biosynthesis in a Deletion laeA Genetic Background

Since VeA and LaeA proteins can interact in the nucleus and are, at least in part, functionally dependent, we examined whether loss of mtfA results in rescue of ST production in a ΔlaeA strain. For this purpose, double ΔmtfAΔlaeA mutants were generated in veA1 and veA+ genetic backgrounds by meiotic recombination from crosses between RJW34-1 (pyrG89; wA3; ΔstcE::argB; ΔlaeA::methG; trpC801; veA1) and TRVp
mtfA (Table 1). Our TLC analysis showed that deletion of mtfA did not recover ST biosynthesis in the strains with laeA deletion (Figure S5).

mtfA Positively Regulates PN Biosynthesis by Controlling the Expression of the PN Gene Cluster

Results from our chemical analysis indicated that mtfA also affects the synthesis of other metabolites (Figure 5). Based on this finding, we also examined whether mtfA controls PN biosynthesis. We evaluated the production of this antibiotic in TRVpΔmtfA and

Figure 8. MtfA localizes in nuclei. A) Diagram of the strategy utilized to fuse GFP to MtfA. The tagged construct was introduced at the mtfA locus by a double-over event. B) Micrographs showing the subcellular localization of the MtfA::GFP in A. nidulans growing in the light or in the dark. Scale bar represents 10 micrometers. doi:10.1371/journal.pone.0074122.g008

Figure 9. Deletion of mtfA affects fungal growth and colony pigmentation. A) Wild type (WT) veA+ (TRV50.2), ΔmtfA (TRVpΔmtfA) and ΔmtfA-com complementation (TRVΔmtfA-com) were point inoculated on GMM plates and incubated at 37°C in either dark or light for 6 days. B) Fungal growth was measured as colony diameter. Values are means of four replicates. Standard error is shown. doi:10.1371/journal.pone.0074122.g009
compared it with PN levels in the isogenic wild-type control and complementation strain. We used a strain of *B. calidolactis* as testing organism. Deletion of *mtfA* decreases penicillin production approximately 7-fold with respect to the wild type (Figure 5A), indicating that *mtfA* is necessary for wild-type levels of penicillin biosynthesis. Our gene expression analysis revealed that *acvA*, *ipnA* and *aatA*, genes in the PN gene cluster [17], are down-regulated in the *mtfA* deletion mutant (Figure 5B–C), particularly at the 24 h time point (24 h after mycelium is transferred to PN induction medium).

Over-expression of *mtfA* clearly increases production of PN (approximately 5-fold) with respect to the PN production levels...
obtained in the wild-type strain (Figure 6A). Expression of acrA, spoA, and outA, was greater in the mtfA over-expression strain than in the control strain (Figure 6B-C). The experiment was repeated several times with similar results.

**mtfA Regulates the Expression of Terrequinone Genes**

We also tested whether mtfA controls the expression of genes involved in terrequinone biosynthesis, a compound known for its anti-tumoral properties [15]. Specifically we examined the expression of tdIA and tdIB [16,55]. At 24 h and 48 h of incubation, expression of tdIA and tdIB was detected in the wild-type control and complementation strains, while transcripts of these genes were absent in the mtfA deletion mutant (Figure 7A). Similarly to the case of ST production, over-expression of mtfA negatively affected the expression of tdIA and tdIB (Figure 7B). Although transcripts were detected for both genes in the mtfA over-expression strain, tdIA expression levels were drastically reduced compared with the control at both 24 and 48 h after induction, and tdIB expression was only detected at 24 h in the over-expression mtfA at very low levels, while it was clearly detectable in the control strain at both time points analyzed (Figure 7B).

**MtfA Subcellular Localization**

We further studied the function of the *A. nidulans* mtfA gene product by examining its subcellular localization in both light and dark conditions. Because the predicted MtfA has a C2H2 DNA binding domain we predicted that it could be found in nuclei. We generated a strain containing MtfA fused to GFP. Our observations using fluorescence microscopy indicated that indeed MtfA localizes in nuclei, as revealed when compared with DAPI staining. Nuclear localization of MtfA was independent of the presence or absence of light (Figure 8).

**mtfA Regulates Asexual and Sexual Development in *A. nidulans***

Deletion of mtfA results in slightly smaller colonies than the wild-type (Figure 9), indicating that mtfA positively influences fungal growth in both light and dark conditions. The mtfA deletion colonies presented a brownish pigmentation which is absent in the control strain. mtfA was expressed at similar levels under conditions promoting either asexual or sexual development, increasing transcript accumulation over time (Figure S6). Conidiophore formation and conidial production was drastically reduced in the mtfA deletion strains with respect to the wild type (Figure 10). This effect was observed in both light and dark cultures. The differences in conidiation levels were more pronounced in the light, a condition that promotes asexual development in *A. nidulans* [56]. In the deletion, the conidiophores produced by the ΔmtfA strain presented fewer metula and phialides than the control strains (Figure S7A). The reduction in conidiation observed in ΔmtfA coincided with alterations in the expression of brlA (Figure 10C), a key transcription factor in the initiation of conidiophore formation [57]. Reduction in brlA expression was observed after 48 h of incubation in the light, condition that promotes conidiophore formation. In the dark brlA levels in the wild type were low, as expected. However, expression of this gene in the mtfA mutant was abnormally high in the dark, a condition that represses conidiation [56]. The increase of brlA expression in ΔmtfA in the dark not only did not result in hyperconidiation, but the conidial production was as low as that observed in ΔmtfA growing in the light.

 Sexual development is also influenced by mtfA. Absence of mtfA in *A. nidulans* results in a more 2-fold reduction in Hulle cells, nursing cells participating in the formation of cleistotheca (fruiting bodies) (Figure 10D) [56]. Cleistothecial production was delayed and decreased in this mutant (Figures 10A, 10E and S7B-C). The cleistothecia present in ΔmtfA were of reduced size (Figure 10A). Expression of ndtD and sttA, encoding transcription factors necessary for the activation of sexual development in *A. nidulans* [58,59] did not significantly change in the absence of mtfA under the experimental conditions assayed (data not shown). Complementation of the deletion mutant with the mtfA wild-type allele restored wild-type morphogenesis.

**Discussion**

This study revealed and characterized a new putative C2H2 transcription factor, MtfA. This protein, located in the cell nuclei, acts as master regulator in the production of several important secondary metabolites. In addition to this role, MtfA also affects asexual and sexual development in *A. nidulans*. MtfA presents two C2H2 zinc finger DNA-binding domains at the C-terminal region. In *A. nidulans* these C2H2 zinc finger domains have been found previously in other regulatory proteins, such as BrlA [57], SteA [60], PacC [52], SltA [61], CrzA [62], CreA [63] and FbC [64]. Of the *A. nidulans* C2H2 zinc finger DNA-binding domain transcription factors examined, MtfA showed the highest similarity to FbC with 25.3% identity. Our *in silico* analysis revealed that MtfA orthologs are present in many filamentous fungi, and they are not found in *S. cerevisiae* or in higher eukaryotes.

Our study indicated that *A. nidulans* MtfA controls the expression of acrR, a gene encoding another transcription factor specifically necessary for the activation of the ST gene cluster [10,11,12], and therefore, affecting the production of the ST toxin. We observed that either absence of mtfA or forced over-expression of mtfA results in a reduction of acrR transcription and decrease in ST biosynthesis, suggesting that only wild-type levels of mtfA gene product, in a balanced stoichiometry with other present factors, is conducive to normal ST levels. This delicate balance among regulatory factors has been previously observed with other regulators. For instance, in case of the global regulator VeA, where both deletion or over-expression of the gene encoding this protein were detrimental to the biosynthesis of the antibiotic PN [21,65]. In addition, our study indicated that the mtfA role in regulating ST production is vea-dependent, which could, at least in part, explain the existence of these biological thresholds for proper function in the case of MtfA abundance in the cell.

VeA has been shown to be functionally associated with LaeA, a chromatin remodeling putative methyltransferase [39,66], that forms part of the velvet complex in the nucleus [36,37]; however, our study showed that deletion of mtfA did not suffice to rescue toxin production in strains where laeA is absent, in both veaI and vea+ genetic backgrounds. This indicates that both laeA and mtfA are necessary for normal ST production in either vea+ or veaI background. Similar results were also observed in the case of the *A. nidulans* rtfA deletion mutant [40], suggesting that although VeA and LaeA are partially functionally connected, they also present differences in their regulatory output. It is possible that mtfA function could be associated with other components of the velvet complex. Future studies in our laboratory will provide further insight on mtfA mechanism of action and its possible connections with other known genetic regulatory networks.

Interestingly, mtfA showed a broad effect influencing the expression of other secondary metabolism gene clusters. Our results revealed that mtfA affects the expression of genes in the terrequinone gene cluster. In this case also both deletion or over-expression of mtfA lead to a reduction in the expression of tdIA and tdIB, that encode asterriquinone synthetase and a protein with
Hülle cells are thick-walled cells that nourish cleistothecial cell was decreased in the mtfA deletion strain. This is relevant since PN production with respect to the wild type, leading to a notable decrease in conidial production in both light and dark cultures, showing accumulation of ST in these cultures and corresponding densitometry is also shown. 

In conclusion, we found a novel master transcription factor, MtfA, that controls the activation of several secondary metabolism gene clusters and regulates asexual and sexual morphological differentiation. MtfA deletion strain resulted in a reduction of PN production, only alRT branch support values >80% are indicated. The protein sequences used are as follows: Aspergillus oryzae (A.oryzae), Aspergillus flavus (A.flavus), Aspergillus niger (A.niger), Aspergillus terreus (A.terreus), Neurospora crassa (N.crassa), Aspergillus fumigatus (A.fumigatus), Aspergillus clavatus (A.clavatus), Aspergillus nidulans (A.nidulans), Penicillium chrysogenum (P.chrysogenum), Penicillium marneffei (P.marneffei), Ajellomyces capsulatus (A.capsulatus), Uncinocarpus ressi (U.ressi), Coccidioides immitis (C.immitis), Fusarium oxysporum (F.oxysporum), Magnaporthe oryzae (M.oryzae), Neurospora tetrasperma (N.tetrasperma), Neurospora crassa (N.crassa), Chaetomium globosum (C.globosum) and Botrytis fuckeliana (B.fuckeliana), NCBI (National center for Biotechnology Information) accession numbers for all sequences utilized in these analyses are shown in Table S1 in the supplemental material. 

Supporting Information

Figure S1 Alignment of MtfA-like proteins in filamentous fungi. Aspergillus nidulans (Anidulans), Aspergillus oryzae (A.oryzae), Aspergillus niger (A.niger), Aspergillus kawachi (A.kawachi), Neurotatorya fischeri (N.fischeri), Penicillium chrysogenum (P.chrysogenum), Coccidioides immitis (C.immitis), Ajellomyces capsulatus (A.capsulatus), Uncinocarpus ressi (U.ressi), Penicillium marneffei (P.marneffei), Botrytis fuckeliana (B.fuckeliana), Neurospora tetrasperma (N.tetrasperma), Neurospora crassa (N.crassa), Magnaporthe oryzae (M.oryzae), Chaetomium globosum (C.globosum) and Fusarium oxysporum (F.oxysporum). Accession ID’s were utilized for alignment and presentation. (RTF)
**Figure S6** Expression of *mtfA* in the wild-type strain. qRT-PCR analysis showing *mtfA* expression in the wild-type strain (*TRV50.2*) at the times indicated under conditions promoting asexual (light) or sexual development (dark). The strains were top-agar inoculated on GMM and incubated at 37°C. (TIF)

**Figure S7** Micrographs of asexual and sexual structures. A) Conidiophores forming in wild type (WT) *zi+z* (*TRV50.2*), ∆*mtfA* (*TRVp∆mtfA*) and ∆*mtfA*-com complementation (*TRV∆mtfA-com*) strains in top agar-inoculated solid GMM cultures incubated for 5 days in the light at 37°C. Bar represent 20 micrometers. CP, conidiophores. B) Micrographs showing the presence of cleistothecia (CL) in wild type (WT) *zi+z* (*TRV50.2*), and ∆*mtfA*-com complementation (*TRV∆mtfA-com*) cultures growing in the dark for 5 days. Magnification 50×. C) Micrographs showing details of sexual structures. Bar represents 15 micrometers. CL, portion of an open cleistothecium; AS, ascospores; HC, Hulle cells. (TIF)

**Table S1** Amino acid sequence comparison of *Aspergillus nidulans* MtfA in with putative orthologs in other fungal species. The comparisons were done using the BLASTp tool provided by NCBI (National Center for Biotechnology Information) and EMBOSS Needle - Pairwise Sequence Alignment tool provided by EMBL-EBI (European Bioinformatics Institute).

**Table S2** Comparison of MtfA with other *A. nidulans* C2H2 transcription factors.

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**Author Contributions**
Conceived and designed the experiments: AMC. Performed the experiments: VR SD AK XF SS AMC. Contributed reagents/materials/analysis tools: AMC. Wrote the paper: AMC VR.
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