INTRODUCTION

Fusarium verticillioides (teleomorph Gibberella moniliformis) is a pathogen of maize that causes severe ear and stalk rots, and it is ubiquitous wherever the crop is grown (Munkvold & Desjardins, 1997). The fungus, a heterothallic ascomycete, can rapidly reproduce and disperse by asexual spores, e.g., macroconidia and microconidia (Desjardins, 2003; Leslie & Summerell, 2006). Attention to diseases caused by F. verticillioides has recently increased due to fumonisins, a group of polyketide-derived mycotoxins structurally similar to sphinganine (Wang et al., 1991). Fumonisin B1 (FB1), the predominant fumonisin found in nature, is highly toxic to human and animals when ingested, and has been linked to oesophageal cancer and neural tube defect in humans, as well as numerous illnesses in animals (Gelderblom et al., 1988; Marasas et al., 2001; Nelson et al., 1993). Microbial pathogens, including F. verticillioides, must recognize and adapt to changes in the extracellular environment during infection and colonization of the host. To accomplish this, pathogens coordinate a complex network of signal transduction pathways that transmit cues from the extracellular environment to the cell and intricately regulate the differential expression of genes critical for adaptation to these changes (for a review see Calvo et al., 2002; Lengeler et al., 2000; Woloshuk & Shim, 2013).

When signalling pathways relay particular external cues to the nucleus, transcriptional activation or suppression of downstream target genes is accomplished by transcription factors (TFs) (Barrera & Ren, 2006; Heintzman & Ren, 2007). Interactions between a TF and DNA sequences are contingent on the sequence-specific or general recognition motifs of binding elements (Barrera & Ren, 2006; Heintzman & Ren, 2007). TF–DNA interactions add complexity and specificity to changes of gene expression in eukaryotic cells. In particular, sequence-specific TFs bind to the regulatory element of the target gene and trigger chromatin reorganization, which is often needed for the pre-initiation complex (i.e. general TFs and RNA polymerase II) to bind to the promoter and activate transcription (Heintzman & Ren, 2007; Lemon & Tjian, 2000), and are generally responsible for the spatial and temporal regulation of transcription and constitute critical components of the signal transduction pathways that regulate a wide spectrum of biological activities in the cell.
The MADS-box family of TFs was first identified through protein sequence homology analysis with diverse eukaryotic organisms (Norman et al., 1988; Passmore et al., 1989; Schwarz-Sommer et al., 1990; Sommer et al., 1990; Yanofsky et al., 1990). These TFs share conserved DNA-binding and dimerization domains, and are involved in the regulation of important cellular processes (Mead et al., 2002; Nolting & Poggeler, 2006a). The MADS-box motif is a highly conserved 56–60 aa region found within the DNA-binding domain; nine of the amino acids are identical in all members described so far. The MADS-box motif generally binds to the consensus sequence CC(A/T)6GG (Shore & Sharrocks, 1995), and is also responsible for the nuclear localization, DNA-binding specificity, accessory factor binding and dimerization of the protein (De Bodt et al., 2003; Messenguy & Dubois, 2003).

Due to their ability to form a dimer, pinpointing a single functional role of MADS-box TFs is not trivial (Shore & Sharrocks, 1995). The N terminus of the protein, which often harbours the MADS-box motif, is the determinant of DNA-binding capabilities, whereas the C terminus is usually involved in dimerization specificity between proteins (Messenguy & Dubois, 2003; Shore & Sharrocks, 1995). Due to their ability to associate with diverse TF types and to form multicomponent regulatory complexes, MADS-box TFs are an intriguing group of proteins involved in the regulation of gene expression in response to different stimuli (Messenguy & Dubois, 2003). Based on the localization of the MADS-box motif, this TF family is often divided in two main groups or types, I and II, with both groups found in plants, animals and fungi (Shore & Sharrocks, 1995). Type I proteins do not share sequence similarity with type II proteins other than the MADS-box motif, and functional studies across these two groups have not been conducted in plants nor other kingdoms (De Bodt et al., 2003). Nevertheless, the type II subfamily has been widely studied in plants, and its members are referred to as MIKC-type proteins. These proteins possess three functional domains in addition to the MADS-box (M) domain: a semi-conserved intervening (I) domain that acts as key regulatory determinant for selection of DNA-binding dimers; a well-conserved keratin (K) domain responsible for dimerization; and a variable C-terminal (C) domain involved in tertiary and quaternary structure formation, as well as transcriptional activation and functional specificity (De Bodt et al., 2003). In animals and fungi, the type II MADS-box TFs are known as MEF2-like (from myocyte-specific factor 2) proteins and bind in a homodimer or heterodimer fashion to the CTA(A/T)4TGA consensus binding sequence (Wu et al., 2011). Meanwhile, CC(A/T)6GG (the CArG box) is the preferred binding consensus sequence for the homodimer-specific SRF-like type I MADS-box TFs (Messenguy & Dubois, 2003; Wu et al., 2011).

The MADS-box TFs have been extensively studied in plants and animal, but only a limited number have been characterized in filamentous fungi. In Saccharomyces cerevisiae, the deletion of RML1 enhanced resistance to cell wall disruptants while reducing cellular flocculation (Dodou & Treisman, 1997). Aspergillus niger RnlA, a Rml1 homologue, is required for cell wall reinforcement during stress (Damveld et al., 2005). The other MADS-box TF in Sacch. cerevisiae, Mcm1, is involved in cell viability and mating (Mead et al., 2002), and a homologue of this TF has been found in Sordaria macrospora (Nolting & Poggeler, 2006b). We postulate that MADS-box TFs contribute to the ability of F. verticillioides to cope with ambient stress factors and to be a successful pathogen. Here we describe our efforts to characterize the role of two MADS-box TFs, designated Mads1 and Mads2, in F. verticillioides.

METHODS

Fungal strains, culture media and growth conditions. Wild-type F. verticillioides strains 7600 and 7598 (Fungal Genetics Stock Center, Kansas City, MO, USA) (Shim & Woloshuk, 2001; Sagaram & Shim, 2007), as well as mutant strains generated in this study (Table 1), were stored in 30 % (v/v) glycerol at −80 °C. Conidia for use as an inoculum were produced by growing the fungus on V8 agar plates (Stevens, 1974) for 7–10 days at 24 °C, then harvested in sterile water, passed through Miracloth (Calbiochem) and quantified using a haemocytometer. For genomic DNA extraction, strains were grown in YEPD liquid medium (Shim & Woloshuk, 2001) overnight at 24 °C with shaking (150 r.p.m.). Tissue for RNA extraction was grown in defined liquid (DL) medium (Shim & Woloshuk, 2001) at 24 °C on a rotary shaker (150 r.p.m.), and harvested at 4, 6, 8 and 10 days post-inoculation.

Nucleic acid manipulation and quantitative real-time RT-PCR (qRT-PCR). Fungal genomic DNA and total RNA were extracted using the OmniPrep DNA isolation kit (G-Biosciences) and TRIzol reagent (Invitrogen Life Technologies), respectively, following the manufacturers’ protocols. Primers for this study are listed in Table S1, available in Microbiology Online. Southern analyses were performed following the standard protocol described earlier (Sagaram & Shim, 2007), where gene-specific probes were 32P-labelled using the Prime-It random primer labelling kit (Stratagene). For qRT-PCR, RNA was converted into cDNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer’s protocol. qRT-PCR analyses were performed on an Applied Biosystems 7500 Fast Real-Time PCR system using the SYBR Green Dynamo ColourFlash qPCR kit (Thermo Fisher Scientific) with 1 µl cDNA reaction as the template. F. verticillioides β-tubulin-encoding gene (TUB2) was used as the endogenous calibrator.

F. verticillioides molecular manipulation. Gene knockout mutants were generated in strains 7600 (MAT1-1 genotype) and 7598 (MAT1-2 genotype) via homologous recombination with split-marker gene-disruption constructs (Fu et al., 2006). Partial fragments of the hygromycin B phosphotransferase gene (HPH) designated HP (766 bp) and PH (924 bp), or the geneticin (G418 sulfate)-resistance gene (GEN) designated GE (1089 bp) and EN (1123 bp), were fused to the 5′ and 3′ flanking regions of the gene of interest via joint-PCR or restriction-enzyme-mediated fusion using primers listed in Table S1. Additional details of construct preparation are provided in Fig. S1. F. verticillioides protoplasts were prepared and transformed as described previously (Sagaram & Shim, 2007; Shin et al., 2013). Transformants were regenerated and selected on regeneration medium containing hygromycin and/or geneticin (150 µg ml−1), depending on the marker used in the cassettes introduced into the transformants.
Maize pathogenicity, FB1 and ergosterol assays. Stalk rot pathogenicity assay was performed on 8-week-old B73 maize plants following the protocol previously described (Sagaram & Shim, 2007; Shim & Woloshuk, 1999). Three maize plants were inoculated per fungal strain, with each plant being inoculated at three internodal regions. For the seed colonization assay, B73 maize seeds were surface sterilized and placed in 100 mm diameter autoclaved glass Petri plates containing a water-soaked 90 mm filter paper (Whatman International). Seeds were inoculated with a suspension of $1 \times 10^5$ conidia or water at the embryo region after making a wound with a sterile needle. Seeds were then incubated at 24°C with a 14 h light/10 h dark cycle and photographed at 7 days post-inoculation.

For FB1 and ergosterol extraction, cracked corn (1 g) was placed in glass vials, rehydrated in water overnight and then autoclaved. Each vial was then inoculated with $1 \times 10^6$ spores of wild-type or mutant strains and placed in an incubator for 10 days at 24°C with a 14 h light/10 h dark cycle. Acetonitrile/ water (1:1, v/v) and chloroform: methanol (2:1, v/v) were added (5 ml) to the vials and incubated at room temperature overnight without agitation for FB1 and ergosterol extraction, respectively. For purification, FB1 crude extracts were passed through equilibrated HyperSep C18 SPE columns (Thermo National). Seeds were inoculated with a suspension of 10^5 conidia in 7600°C with a 14 h light/10 h dark cycle and photographed at 7 days post-inoculation.

For FB1 and ergosterol extraction, cracked corn (1 g) was placed in glass vials, rehydrated in water overnight and then autoclaved. Each vial was then inoculated with $1 \times 10^6$ spores of wild-type or mutant strains and placed in an incubator for 10 days at 24°C with a 14 h light/10 h dark cycle. Acetonitrile/ water (1:1, v/v) and chloroform: methanol (2:1, v/v) were added (5 ml) to the vials and incubated at room temperature overnight without agitation for FB1 and ergosterol extraction, respectively. For purification, FB1 crude extracts were passed through equilibrated HyperSep C18 SPE columns (Thermo Fisher Scientific) and ergosterol crude extracts were passed over Acrodisc 13 mm nylon 0.45 μm filters ( Pall Life Sciences). HPLC analyses of FB1 and ergosterol were performed as described elsewhere (Shim & Woloshuk, 1999; Kim et al., 2011). FB1 levels were then normalized to ergosterol contents. The experiment was repeated twice with at least three biological replicates.

Conidia production, vegetative growth and sexual mating assays. V8 agar, defined medium agar (Shim & Woloshuk, 2001) and 0.2× potato dextrose agar (PDA) plates were inoculated with a 10 μl of $1 \times 10^6$ spore suspension and incubated at 24°C with a 14 h light/10 h dark cycle. We measured and recorded radial colony growth on V8, 0.2× PDA and defined medium plates at 4 days post-inoculation. For conidia production, defined medium plates were incubated for 7 days. Once time had passed, 5 ml sterile water was added to each plate and conidia were resuspended by scraping the colony surface with a sterile spreader. Conidia were counted using a haemocytometer.

Sexual mating experiments were performed as described previously (Sagaram et al., 2007). All strains were first grown on V8 agar plates, and subsequently strains used as maternal parents were transferred to carrot agar plates. Cultures were incubated for 7 days at 24°C with a 14 h light/10 h dark cycle. Subsequently, conidia of the strains used as the paternal parent were harvested from V8 agar plates and applied to carrot agar cultures of the maternal parent strains. All strains generated in this study were tested as paternal and maternal parents with wild-type strains serving as controls. Crosses were incubated under standard F. verticillioides mating conditions until perithecia and cirri were observed.

RESULTS

F. verticillioides contains two putative MADS-box TFs

We screened for putative MADS-box TFs in F. verticillioides by selecting the following list of known fungal MADS-box TFs and subsequently investigating the Fusarium comparative database (Ma et al., 2010): Mcm1 from Sordaria macrospora (XP_003347020.1) and Sacch. cerevisiae (CAA88409), as well as Mig1 from Magnaporthe grisea (ABX79379.1), RmlA from A. niger (XP_001400349.1) and Rml1 from Sacch. cerevisiae (NP_015236.1). Using the BLASTP algorithm, we isolated two putative MADS-box TFs in F. verticillioides: a 222 aa protein designated Mads1 (FVEG_01965) encoded by a 2056 bp gene interrupted by two introns (278 and 71 bp), and a 652 aa protein designated Mads2 (FVEG_03759) encoded by a 2114 bp gene with two introns (67 and 71 bp). A study of the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/), revealed that the Mads1 protein contains a SRF-like/type I MADS-box domain (Fig. S1), whereas the Mads2 protein contains a MEF2-like/type II MADS-box domain (Fig. 1b). These two subfamilies differ mainly in the alpha 2 helix responsible for dimerization (Messengu & Dubois, 2003), and these domains are known as SAM and MEF2 domains (Fig. 1c, d).

Generation of MADS1 and MADS2 deletion mutants and complements

To characterize the function of the two MADS-box TFs in F. verticillioides, we generated gene-deletion mutants Fmt1 (Δmads1::HPH) and Fmt2 (Δmads2::HPH) in F. verticillioides 7600 (MAT1-1) strain (Fig. S1). In addition, using the same approach we generated Fmt4 (Δmads1::GEN) and

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>7600</td>
<td>F. verticillioides wild-type strain</td>
<td>MAT1-1</td>
<td>MADS1 MADS2*</td>
</tr>
<tr>
<td>7598</td>
<td>F. verticillioides wild-type strain</td>
<td>MAT1-2</td>
<td>MADS1 MADS2*</td>
</tr>
<tr>
<td>Fmt1</td>
<td>Deletion of MADS1 in 7600</td>
<td>MAT1-1</td>
<td>Δmads1::HPH†</td>
</tr>
<tr>
<td>Fmt2</td>
<td>Deletion of MADS1 in 7600</td>
<td>MAT1-1</td>
<td>Δmads2::HPH†</td>
</tr>
<tr>
<td>Fmt3</td>
<td>Deletion of MADS1 in Fmt2</td>
<td>MAT1-1</td>
<td>Δmads1::GEN, Δmads2::HPH†</td>
</tr>
<tr>
<td>Fmt4</td>
<td>Deletion of MADS1 in 7598</td>
<td>MAT1-1</td>
<td>Δmads1::GEN†</td>
</tr>
<tr>
<td>Fmt5</td>
<td>Deletion of MADS1 in 7598</td>
<td>MAT1-2</td>
<td>Δmads2::GEN†</td>
</tr>
<tr>
<td>Fmt6</td>
<td>Complementation of Fmt1</td>
<td>MAT1-1</td>
<td>Δmads1::HPH, MADS1::GEN†</td>
</tr>
<tr>
<td>Fmt7</td>
<td>Complementation of Fmt2</td>
<td>MAT1-1</td>
<td>Δmads2::HPH, MADS2::GEN†</td>
</tr>
</tbody>
</table>

*Strain source: Fungal Genetics Stock Center.
†Strains generated in this study.

Table 1. Fungal strains used in this study
Fmt5 (Δmads2::GEN) in F. verticillioides 7598 (MAT1-2) strain. We also generated a double-deletion mutant, Fmt3 (Δmads2::HPH, Δmads1::GEN), in the F. verticillioides 7600 strain (Table 1). These mutations were first screened by PCR (data not shown). Subsequent confirmation by Southern blot (Fig. S2), where DNA samples were treated with NcoI, showed the wild-type (7600) strain had a 605 bp band, while the deletion strain Fmt1 had a 1025 bp band, indicating the replacement of MADS1 gene with the knockout construct. In the case of MADS2, after SphI-DNA digestion, the wild-type (7600) strain yielded two bands (2945 and 1550 bp), whereas the knockout mutant only yielded a compound 1600 bp band (1550 and 1650 bp fragments), confirming the replacement of the MADS2 gene with the HPH gene. However, in strain 7598, the wild-type showed a 1786 bp band, whereas the strain Fmt4 showed a 2577 bp band, indicating replacement of MADS1 with the GEN gene. This same construct and strategy was used to generate the Fmt3 strain. Strains Fmt1, Fmt2, Fmt4 and Fmt5 were complemented by reinserting the respective wild-type gene along with a GEN or HPH marker. Single insertion of the complementation construct in each strain was confirmed by Southern blot (Fig. S2).

Fmt1 mutant exhibits a pleiotropic vegetative phenotype whereas Fmt2 is indistinguishable from the wild-type strain

To assess the role of MADS-box TFs in the development of F. verticillioides, we monitored the growth of gene-deletion mutants and wild-type strains on V8 agar, 0.2× PDA and defined medium plates (Fig. S3). When colony growth was measured after a 4 day incubation at 24 °C with a 16/8 h light/dark cycle on defined medium and PDA, Fmt1, Fmt2 and Fmt3 strains showed reduced colony growth when compared to the wild-type progenitor (P<0.05). Notably, when these mutants were grown on defined medium modified with sorbitol no significant growth defect was observed when compared to standard defined medium (data not shown). When conidiation levels were normalized to growth on defined medium, we did not observe a statistical difference between the wild-type and mutant strains (Table 2). In addition, we observed the production of an eggplant-purple...
secondary metabolite in Fmt1 when the strain was grown on V8 agar (Fig. 2) and 0.2 × PDA, but not in the wild-type or other mutant strains. In particular, it was inexplicable why this pigment was not observed in Fmt4, a MADS1 deletion generated in strain 7598. Furthermore, these mutants seemed to rather generate colonies with a higher density of aerial mycelia (Fig. 2), but this was not easily quantifiable.

### MADS-box TF-encoding genes play a key role in *F. verticillioides* mycotoxin biosynthesis but not virulence

Maize stalk and seed colonization assays were performed with *F. verticillioides* wild-type and mutant strains to test whether MADS1 and MADS2 genes play a role in virulence. Maize stalks (8 weeks old) and B73 seeds were inoculated with fungal spores (1 × 10^5) or water (negative control) as described by Sagaram & Shim (2007). Subsequently, stalks were split longitudinally in half with a scalpel to assess disease severity (Fig. 3a), while seeds were deemed colonized by observing the presence of fungal mycelia on the kernel surface (Fig. 3b). All strains successfully colonized maize kernels and stalks, and these observations led us to conclude that the mutant strains are equally capable of colonizing and causing disease in maize stalk and kernel when compared to the wild-type strain. However, when these mutant strains were analysed for their ability to produce FB1, we saw drastic differences.

### Table 2. Growth and conidia production

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony diameter (mm)*</th>
<th>10^−4× Conidiation†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Defined agar</td>
<td>0.2× PDA</td>
</tr>
<tr>
<td>7600</td>
<td>4.40 ± 0.01</td>
<td>5.00 ± 0.03</td>
</tr>
<tr>
<td>Fmt1</td>
<td>4.10 ± 0.12‡</td>
<td>4.14 ± 0.01‡</td>
</tr>
<tr>
<td>Fmt2</td>
<td>3.48 ± 0.01‡</td>
<td>4.36 ± 0.05‡</td>
</tr>
<tr>
<td>Fmt3</td>
<td>3.44 ± 0.01‡</td>
<td>4.30 ± 0.03‡</td>
</tr>
<tr>
<td>Fmt6</td>
<td>5.08 ± 0.12‡</td>
<td>4.88 ± 0.01</td>
</tr>
<tr>
<td>Fmt7</td>
<td>4.44 ± 0.06</td>
<td>5.22 ± 0.01‡</td>
</tr>
</tbody>
</table>

*Mean and ± values were calculated from the results from five replicates after a 4-day incubation.
†Mean and ± values were calculated from the results from eight replicates grown on defined agar for 7 days.
‡The values measured for the mutants were statistically different from those of the wild-type (7600) when analysed using Student’s t test (P<0.05).

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**Fig. 2.** Colony morphology of *F. verticillioides* wild-type (7600) and mutant (Fmt1–7) strains grown on V8 agar at 24 °C for 10 days: (a) top-surface and (b) cross-section views. The rules on the right in (b) have subdivisions of 1 mm.
**MADS1** is a broad regulator of polyketide synthase (PKS)-derived secondary metabolites

With the observation that FB1 production is negatively affected in the mutant strains, as well as the over-production of a yet-to-be-defined pigment in the Fmt1 strain, we decided to investigate the expression of 15 putative PKS genes in *F. verticillioides* by qRT-PCR. Knockout and wild-type strains were grown in a DL medium known to support the production of FB1 (Shim & Woloshuk, 2001). Tissue samples were harvested at 6, 8 and 10 days post-inoculation, and total RNA was extracted. PKS expression levels in the wild-type strain were standardized to 1.0 and used as a reference. Additionally, β-tubulin expression levels were used as endogenous normalizer in all samples. At 6 days post-inoculation, the expression levels of all PKS genes in Fmt1, except *PKS10*, were similar to or surpassed the levels seen in the wild-type (Table S3). This trend tailed off with time, and at 8 days post-inoculation the expression levels of only *PKS8, PKS13* and *PKS15* genes in Fmt1 were higher than those in the wild-type strain (Table S4). Interestingly, at 10 days post-inoculation, *PKS10* was the only gene in Fmt1 with expression levels similar to the wild-type strain (Table 3). Transcript levels of the remaining 14 genes decreased by at least half when compared to those of the wild-type.

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**Table 3.** Transcription levels of PKS genes at 10 days post-inoculation

Total RNA samples were prepared from fungal strains grown in defined medium. qRT-PCR analysis of gene expression was performed with SYBR-Green as the fluorescence reporter. Expression of each gene was normalized to the gene expression of endogenous β-tubulin (TUB-2) (GenBank accession no. U27303), and was calibrated using $2^{-\Delta\Delta CT}$. Data represent the fold difference where the wild-type level is standardized to 1.0 and SE is presented. Each value is the mean of at least three biological replicates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene expression per strain</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT 7600</td>
</tr>
<tr>
<td>PKS1</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>PKS2</td>
<td>1.0 ± 0.06</td>
</tr>
<tr>
<td>PKS3</td>
<td>1.0 ± 0.06</td>
</tr>
<tr>
<td>PKS4</td>
<td>1.0 ± 0.12</td>
</tr>
<tr>
<td>PKS5</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>PKS6</td>
<td>1.0 ± 0.09</td>
</tr>
<tr>
<td>PKS7</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>PKS8</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>PKS9</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>PKS10</td>
<td>1.0 ± 0.08</td>
</tr>
<tr>
<td>PKS11</td>
<td>1.0 ± 0.07</td>
</tr>
<tr>
<td>PKS12</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>PKS13</td>
<td>1.0 ± 0.14</td>
</tr>
<tr>
<td>PKS14</td>
<td>1.0 ± 0.06</td>
</tr>
<tr>
<td>PKS15</td>
<td>1.0 ± 0.08</td>
</tr>
</tbody>
</table>

Fmt1 strain produced 50% less FB1 than the wild-type strain, while the Fmt2 and Fmt3 strains produced 80% less FB1 when compared to the wild-type (Fig. 3c). These results suggest that **MADS1** and **MADS2** play a critical role in FB1 biosynthesis, but are not involved in *F. verticillioides* virulence.
However, theFmt2 strain produced PKS transcript levels similar to the wild-type strain throughout the study.

**Gene knockout mutations in the *F. verticillioides* 7598 (MAT1-2 genotype) strain render the organism unable to produce perithecia and ascospores**

Nolting & Pöggeler (2006a) have shown that SmMcm1, a MADS-box TF in *Sorda. macrospora*, plays an important role in sexual reproduction. *In silico* analysis showed that *F. verticillioides* Mads1 shares 74% similarity with SmMcm1 at the amino acid level. Thus, we reasoned that the functional role of MADS-box TF in *Sorda. macrospora*, namely in sexual development, is conserved in the heterothallic ascomycete *F. verticillioides*. Using the gene-deletion mutants generated in two different mating types, we performed sexual mating assays and found that mutants generated in the 7598 background failed to produce perithecia when sexually crossed with the wild-type strain 7600 or Δmads mutant strains derived from it (Fig. 4). However, mutants derived from the 7600 background were able to produce perithecia and ascospores when crossed with the wild-type strain 7598, but not with mutant strains derived from it (Table S2). Therefore, our data suggest that MADS-box TFs are required for the sexual mating potential in MAT1-2 strains, but are dispensable in MAT1-1 strains.

**DISCUSSION**

MADS-box TFs are involved in a wide array of biological activities (Shore & Sharrocks, 1995). Four genes encoding MADS-box TFs are found in the *Sacch. cerevisiae* genome (Alvarez-Buylla *et al.*, 2000; Ma *et al.*, 2010), while only two were found in *F. verticillioides*. Mads1, a SRF-type MADS-box protein, is homologous to *Sacch. cerevisiae* and *Sorda. macrospora* Mcm1, while Mads2, a MEF-2-type MADS-box protein, is homologous to *Sacch. cerevisiae* Rml1 and A. niger RmlA. Studies of the Mcm1 homologues in *Sorda. macrospora*, *Magnaporthe oryzea* and *Ustilago maydis* have further demonstrated the role of SRF-type MADS-box proteins in sexual mating and virulence in filamentous fungi (Krüger *et al.*, 1997; Nolting & Pöggeler, 2006a; Zhou *et al.*, 2011). Studies of *Sacch. cerevisiae* Rml1 and A. niger RmlA proteins have shown that MEF2-type MADS-box proteins are one of the targets of a Pkc1-controlled mitogen-activated protein kinase (MAPK) cascade. Thus, Rml1 and RmlA have been associated with the cell wall integrity MAPK pathway, with a key role in reinforcement and remodelling of the cell wall upon extracellular stress (Damveld *et al.*, 2005; Dodou & Treisman, 1997; Levin, 2005). Here, we used *F. verticillioides*, a major pathogen of maize worldwide, to further investigate the role of MADS-box TFs in secondary metabolite biosynthesis, sexual development and virulence.

We performed virulence assays where host material was inoculated with spores of wild-type or mutant strains. The result showed that MAD1 and MAD2 genes in *F. verticillioides* are not essential for infestation or colonization of maize stalks and kernels. A similar observation was reported in the basidiomycete *U. maydis*, where a mutation in the umc1 gene did not affect the virulence of the fungus when inoculated on maize kernels (Krüger *et al.*, 1997). However, MADS-box TF-encoding genes in the rice pathogen *M. grisea* have been shown to be required, if not essential, for the ability to colonize the host. The rice blast pathogen infects rice leaves through the formation of a specialized structure called an appressorium. This structure is used to puncture the plant cuticle and cell walls

![Fig. 4. *F. verticillioides* sexual mating assay. Wild-type 7600 (MAT1-1) and mutant strains generated in this background were crossed to strains of the opposite mating type, e.g. wild-type 7598 (MAT1-2). These plates were incubated under conditions described previously by Sagaram *et al.* (2007) until perithecia (indicated by an asterisk) and ascospores were observed. The presence of ascospores in cirri (indicated by an arrow) was confirmed via microscopy.](http://mic.sgmjournals.org)
using turgor pressure, and subsequently differentiates into primary and secondary invasive hyphae as it moves into the host, colonizing the penetrated and neighbouring cells (De Jong et al., 1997; Howard et al., 1991). MoMcm1, an orthologue of F. verticillioides Mads1 and U. maydis Umc1, has been characterized in M. grisea, and it was demonstrated that the mutant is delayed in appressorium formation, and thus is defective in penetration and colonization of the host (Zhou et al., 2011). Additionally, M. grisea Mig1, an orthologue of F. verticillioides Mads2, has been shown to help the fungus overcome plant defences upon colonization. The knockout mutant had a normal growth rate and was able to form appressoria, but was non-pathogenic since the formation of secondary infectious hyphae was blocked. In addition, the mig1 mutant failed to infect rice leaves through leaf wounds (Mehrabi et al., 2008). The mechanism by which F. verticillioides invades plant tissue is largely different from that of M. grisea. The fungus does not have the capabilities to mechanically penetrate plant cuticles as it does not produce appressorium-like structures, and it is only known to colonize plant tissue through mechanical or insect damage (Duncan & Howard, 2010). Therefore, it is not surprising that MADS-box TFs are dispensable for the pathogenicity of F. verticillioides and U. maydis, both non-appressorium-producing organisms, while significantly impacting the ability of M. grisea to penetrate and colonize live rice leaves. In addition, we found no experimental evidence suggesting that F. verticillioides Mads1 and Mads2 are involved in the fungal differentiation in planta, nor in overcoming plant defences upon colonization.

Secondary metabolite production in filamentous fungi is orchestrated by the co-regulation of biosynthetic genes frequently arranged in clusters. F. verticillioides produces FB1 upon colonization of maize kernels. To date, the FB1 biosynthetic gene cluster, known as the FUM cluster, is known to be composed of at least 15 co-transcribed genes, where FUM21 encodes a TF that positively regulates the expression of FUM genes. In particular, FUM1 encodes a PKS responsible for the formation of the linear carbon chain in fumonisins. Nonetheless, a number of regulatory genes outside the cluster have been associated with FB1 biosynthesis (Flaherty et al., 2003; Flaherty & Woloshuk, 2004; Sagaram et al., 2006; Shim & Woloshuk, 2001), demonstrating that non-clustered genes can act as regulators of FB1 synthesis as well. Here, we found that disruption of MADS1 and MADS2 had an adverse effect on FB1 production, as the FB1 levels in the Fmt1 and Fmt2 strains decreased 50 and 80%, respectively, when compared to the wild-type strain. Interestingly, double deletion of MADS1 and MADS2 resulted in similar FB1 levels to those produced by MADS2 deletion alone, suggesting that MADS2 may be the primary MADS-box regulator of FB1 biosynthesis in F. verticillioides.

Notably, fumonisins are secondary metabolites produced by PKSs (Brown et al., 1996; Proctor et al., 1999). Commonly found in bacteria, fungi and plants, PKSs produce a large and structurally diverse group of compounds. In fungi, PKSs are known to produce pigments, cell-to-cell signalling molecules and toxins (Kroken et al., 2003). Fungal PKSs are multifunctional peptides that contain all functional domains necessary to fully form polyketides within one module (Hopwood & Sherman, 1990; Staunton & Weissman, 2001). In silico analysis showed that 15 PKS genes are present in the F. verticillioides genome (Kroken et al., 2003). The disruption of MADS1 not only led to the reduction of FB1 biosynthesis, but also led to the production of a diffusible eggplant-purple pigment that was not observed in the wild-type or Fmt2 strain. To further investigate whether MADS-box TFs influence other secondary metabolites, we followed the expression of the 15 PKS genes present in the F. verticillioides genome via qRT-PCR. We chose modified myro medium as the growth medium to capture FUM1 mRNA in our samples, since the medium has been show to induce FB1 production in F. verticillioides (Shim & Woloshuk, 1999). The results showed that the disruption of MADS1 had a broad negative impact on the expression of all PKS genes when compared to the wild-type, whereas the MADS2 mutation did not. This outcome prompted us to postulate that Mads1 is a broad regulator of secondary metabolism in F. verticillioides, and that it may target regulons upstream of Mads2 to influence FB1 production. Our PKS expression and FB1 production data suggest that while the disruption of MADS2 did not have a significant negative effect on the expression of FUM1 (PKS11), levels of FB1 toxin were significantly lower than those of the wild-type strain. However, disruption of MADS1 led to significantly lower FUM1 expression levels but still produced 50% of the FB1 when compared to the wild-type. Interestingly, the double mutant produced the same amount of FB1 as the MADS2 knockout mutant, which leads us to hypothesize that while MADS1 has a direct impact in FUM1, MADS2 acts downstream in the polyketide biosynthesis pathway playing a role in the later stages of the fuminson molecule synthesis. Further studies of the regulons of MADS-box TFs are needed to test this hypothesis.

Sexual reproduction in fungi is a complex process that requires precise spatiotemporal regulation of mechanisms and molecules (Fraser & Heitman, 2004, 2005; Bloemendal et al., 2012). One of the first MADS-box TFs characterized, Sacch. cerevisiae Mcm1, is known to interact with mating-type proteins (Passmore et al., 1989; Schwarz-Sommer et al., 1990; Johnson, 1995). In U. maydis, deletion of Umc1, an orthologue of Sacch. cerevisiae Mcm1, did not impact viability or fertility of haploid cells, but significantly attenuated the expression of genes involved in pheromone production and receptor genes resulting in the absence of mating hyphae in mating assays (Krüger et al., 1997). The function of Mcm1 orthologues in other fungi has also been tested in Sorda. macrospora and M. oryzae. Nolting & Pöggeler (2006b) showed that Sorda. macrospora was only able to produce protoperithecia and failed to form either
perithecia or ascospores upon deletion of the MCM1 gene. They further demonstrated via yeast two-hybrid and far-western analyses that the MCM1 protein interacts with SMTA-1, which is a protein known to form a heterodimer with SmtA-1, another Soralla macrospora mating-type locus-encoded protein. Such interaction between SmtA-1 and MCM1 is characteristic of mating-type transcriptional regulators in fungi. Similar to the phenotype observed in the mcm1 mutant, the SmtA-1 knockout mutant is sterile as it is not able to develop beyond the protoperithecia formation stage. The authors propose that SmtA-1 may form a complex with SmtA-1 and MCM1, and that MCM1 may play a role in the transcriptional regulation of mating-specific genes and fruiting-body development in Soralla macrospora. In M. oryzae, Zhou et al. (2011) further confirmed via yeast two-hybrid assay the interaction of MCM1 with mating-type loci proteins and reported the sterility of MAT1-1 strains lacking the MoMCM1 gene. In F. verticillioides two mating-type idiomorphs, MAT1-1 and MAT1-2, are recognized. Deletion of either MADS1 or MADS2 in the MAT1-2 genetic background rendered the organism unable to produce perithecia and ascospores, while mutations on the MAT1-1 genotype maintained fertility. This was an intriguing outcome as our results show that both MADS-box TFs only influence one mating type but not the other in sexual recombination. To our knowledge, the role of MADS-box TFs in secondary metabolite biosynthesis or the impact of MADS-box MEF2-like TFs in sexual reproduction have not been previously reported in filamentous fungi. Further studies on the specific targets of MADS-box TFs in F. verticillioides are needed to provide a comprehensive understanding of the role of these proteins in the sexual reproduction and secondary metabolism in the fungus.

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