Mating-Type Genes from Asexual Phytopathogenic Ascomycetes *Fusarium oxysporum* and *Alternaria alternata*

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Mating-type (MAT) loci were cloned from two asexual (mitosporic) phytopathogenic ascomycetes, *Fusarium oxysporum* (a pyrenomycete) and *Alternaria alternata* (a loculoascomycete), by a polymerase chain reaction (PCR)-based strategy. The conserved high mobility group (HMG) box domain found in the MAT1-2-1 protein was used as a starting point for cloning and sequencing the entire MAT1-2 idiomorph plus flanking regions. Primer pairs designed to both flanking regions were used to amplify the opposite MAT1-1 idiomorph. The MAT1-1 and MAT1-2 idiomorphs were approximately 4.6 and 3.8 kb in *F. oxysporum* and approximately 1.9 and 2.2 kb in *A. alternata*, respectively. In both species, the MAT1-1 idiomorph contains at least one gene that encodes a protein with a putative alpha box domain and the MAT1-2 idiomorph contains one gene that encodes a protein with a putative HMG box domain. MAT-specific primers were used to assess the mating type of *F. oxysporum* and *A. alternata* field isolates by PCR. MAT genes from *A. alternata* were expressed. The *A. alternata* genes were confirmed to be functional in a close sexual relative, *Cochliobolus heterostrophus*, by heterologous expression.

Sexual reproduction in ascomycetes is controlled by a single regulatory locus referred to as a mating type (MAT) (Coppin et al. 1997; Turgeon 1998). In heterothallic fungi, the sexual cycle is initiated when strains of opposite mating types interact. MAT loci have been cloned from many heterothallic ascomycetes, including *Saccharomyces cerevisiae* (Hicks et al. 1979), *Schizosaccharomyces pombe* (Kelly et al. 1988), *Neurospora crassa* (Glass et al. 1990; Staben and Yanofsky 1990), *Podospora anserina* (Debuchy and Coppin 1992), *Cochliobolus heterostrophus* (Turgeon et al. 1993), and *Pyrenopeziza brassicae* (Singh and Ashby 1998). The single MAT locus has alternate sequences, termed idiomorphs, which

lack significant sequence similarity to each other (Metzenberg and Glass 1990; Turgeon 1998). All ascomycete MAT idiomorphs encode proteins with confirmed or putative DNA-binding motifs (e.g., MAT1-2 = high mobility group [HMG] box; MAT1-1 = alpha box), which suggests that MAT genes encode transcriptional regulators and control the expression of some genes required for sexual reproduction (Turgeon 1998). In this report, strains carrying the idiomorph that encodes a protein with an alpha box motif are designated MAT1-1 and the opposite are designated MAT1-2.

A large number of phytopathogenic fungi are asexual (mitosporic) species that lack sexual reproduction. Sharon et al. (1996) reported that *Bipolaris sacchari*, an asexual relative of heterothallic *C. heterostrophus* (anamorph *B. maydis*), carries MAT genes. Heterologous expression showed that *B. sacchari* MAT1-2 was functional in *C. heterostrophus* but not in its own genetic background, suggesting that asexuality in this fungus results from the loss of a function other than MAT (Sharon et al. 1996).

Previously it was difficult to determine whether asexual fungi had MAT genes because they could not be crossed with each other. Recently we reported on a polymerase chain reaction (PCR)-based approach to obtain mating type genes from many species and demonstrated that asexual species also carried MAT (Arie et al. 1997). This method was based on the observation that all reported MAT1-2 idiomorphs in heterothallic ascomycetes encode a protein with an approximately 270-bp HMG box motif (Turgeon 1998; Turgeon et al. 1993). Degenerate primers designed to the HMG box domain of *N. crassa* and *C. heterostrophus* were useful to amplify this region from pyrenomycete and loculoascomycete MAT genes, respectively (Arie et al. 1997).

As a first step into learning what causes asexuality in ascomycetes, we determined in this study that asexual phytopathogenic ascomycetes, *F. oxysporum* (a pyrenomycete), and *A. alternata* (a loculoascomycete) have MAT genes. The HMG box motif was used as a starting point to clone the entire MAT1-2 idiomorph as well as the flanking regions by PCR. The flanking regions were then used to clone the MAT1-1 idiomorph on the basis of our assumption that the flanking regions of both idiomorphs should be conserved.

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RESULTS

Cloning mating-type idiomorphs of *F. oxysporum* by a PCR-based approach.

Degenerate primers FHMG11 and FHMG12 (Table 1) corresponding to the HMG box motif were based on the nucleotide sequence data of *Gibberella fujikuroi* (Arie et al. 1999). With the use of these primers, an approximately 190-bp fragment was amplified from the genomic DNA of *F. oxysporum* strain SUF959 (Fig. 1A.1). The remaining *F. oxysporum* strain, 880621a-1, showed no amplification with these primers. The fragment amplified from SUF959 was cloned into the pCR2.1 vector and sequenced. The nucleotide sequence of the fragment was similar to those of the reported HMG box motifs in the MAT1-2 protein of ascomycetes (Turgeon 1998), particularly to that of *G. fujikuroi* MAT1-2 (92.6 and 94.4% in nucleotide and amino acid levels, respectively) (Arie et al. 1999).

The entire MAT1-2 idiomorph and flanking regions were obtained from strain SUF959 by a combination of inverse- and

thermal asymmetric interlaced (TAIL)-PCR (Liu and Whitter 1995; Silver 1991), with the cloned region encoding the HMG box motif as a starting point (Fig. 1A). About 1 µg of genomic DNA of strain SUF959 was digested with 40 U of *Bam*HI and *Eco*RV separately and self-ligated. Long-range PCR was performed with the self-ligated genomic DNA as a template with FHM101 and FHM104 as primers (Table 1 and Fig. 1A.2). For analysis of PCR products, 10 µl of each reaction mixture was subjected to agarose gel (0.8%) electrophoresis in Tris-acetate-EDTA buffer. The inverse-PCR procedure yielded an approximately 3.8-kb *Bam*HI and 2.9-kb *Eco*RV fragment, each of which was sequenced and combined, resulting in an approximately 5.2-kb (*Bam*HI-*Eco*RV) sequence that included the HMG box motif (Fig. 1A.2).

DNA gel blot analysis was used to determine whether the approximately 5.2-kb sequence included flanking regions, which were expected to be common to both mating types. Genomic DNA of SUF959 (amplification of the HMG box motif was observed) and 880621a-1 (no amplification) hy-

Table 1. Primers used for polymerase chain reaction (PCR)

Name	Nucleotide sequence ^a	Comments	Reference
FHMG11	5'-TACCGYAAGGAGCGTCACC	30 to 48 bp at the high mobility group (HMG) box in <i>Gibberella fujikuroi</i> MAT1-2 sequence (accession no. AB005041)	Arie et al. 1999
FHMG12	5'-TTYWYCTSATCSGCSMKHWSCTTG	218 to 196 bp at HMG-box in the <i>G. fujikuroi</i> MAT1-2 sequence	Arie et al. 1999
FHM101	5'-CTTGCCCGCCTCTGGAACCTC	4,339 to 4,358 bp at the <i>Fusarium oxysporum</i> MAT1-2 idiomorph (accession no. AB011378) used for inverse-PCR	This study
FHM104	5'-ATCGAMTGGTGACGCTCCTT	4,245 to 4,226 bp at the <i>F. oxysporum</i> MAT1-2 idiomorph used for inverse-PCR	This study
Fo52	5'-AATTTGGGAAGACGAGAGAGTT	1,872 to 1,853 bp at the <i>F. oxysporum</i> MAT1-2 idiomorph used for the primary cycles of TAIL-PCR	This study
Fo50	5'-CGCAACCTGCTATCATCTCAAA	1,846 to 1,825 bp at the <i>F. oxysporum</i> MAT1-2 idiomorph used for the secondary cycles of TAIL-PCR	This study
Fo25	5'-AATAGCGAACAAGGATCAGGG	Flanking region of MAT loci (671 to 691 bp in MAT1-2; 1 to 21 bp in the MAT1-1 sequence [accession no. AB011379]) of <i>F. oxysporum</i>	This study
Fo14	5'-ATATGGAGCAGTGATTGGAC	At the flanking region of MAT loci (5,122 to 5,103 bp in MAT1-2, 5,220 to 5,199 bp in the MAT1-1 sequence) of <i>F. oxysporum</i>	This study
Falpha1	5'-CGGTCAYGAGTATCTTCCTG	1,575 to 1,556 bp at the <i>F. oxysporum</i> MAT1-1 idiomorph	This study
Falpha2	5'-GATGTAGATGGAGGGTTCAA	1,200 to 1,219 bp at the <i>F. oxysporum</i> MAT1-1 idiomorph	This study
ChHMG1	5'-AAGGCNCCNCGYCCNATGAAC	925 to 945 bp at HMG-box in the <i>Cochliobolus heterostrophus</i> MAT1-2 sequence (accession no. X68398) used for the primary cycles of TAIL-PCR	Arie et al. 1997
ChHMG2	5'-CTNNGNGTGTAATTGTAATTNGG	1,200 to 1,178 bp at HMG-box in the <i>C. heterostrophus</i> MAT1-2 sequence used for the primary cycles of TAIL-PCR	Arie et al. 1997
AaHMG3	5'-TGGATCATCTTTCGAGATGC	1,469 to 1,488 bp at the <i>Alternaria alternata</i> MAT1-2 (accession no. AB009452) idiomorph used for the secondary cycles of TAIL-PCR	This study
AaHMG4	5'-TTCTTTCGCCGACTGTGC	1,678 to 1,661 bp at the <i>A. alternata</i> MAT1-2 idiomorph used for the secondary cycles of TAIL-PCR	This study
AaFS3	5'-AGCCAACAATACCTTCAGTCAG	Flanking region of MAT loci (adjacent to the 5' end of MAT1-2 and MAT1-1 sequence (accession no. AB009451) of <i>A. alternata</i>	This study
AaM1-6	5'-ACCTCCTTACATTTCAACAC	Flanking region of MAT loci (2,988 to 2,959 bp) in MAT1-2; 2,667 to 2,648 bp in the MAT1-1 sequence) of <i>A. alternata</i>	This study
AAM1-1	5'-CAGGTTTTCGTTTCACGGCTC	608 to 627 bp at MAT1-1 of <i>A. alternata</i> used for RT-PCR	This study
AAM1-2	5'-TCCAAACTCGCAGTGGAAG	665 to 684 bp at MAT1-1 of <i>A. alternata</i> used for RT-PCR	This study
AAM1-3	5'-GATTACTCTTCTCCGCAGTG	1,353 to 1,334 bp at MAT1-1 of <i>A. alternata</i> used for RT-PCR	This study
AAM2-1	5'-CTTCGGAACGGGGTTGTGCG	1,219 to 1,238 bp at MAT1-2 of <i>A. alternata</i> used for RT-PCR	This study
AAM2-2	5'-GCTCACACACTCTTGTAAG	1,311 to 1,330 bp at MAT1-2 of <i>A. alternata</i> used for RT-PCR	This study
AAM2-3	5'-TTGGAAATTGAGAACCTCCG	1,807 to 1,788 bp at MAT1-2 of <i>A. alternata</i> used for RT-PCR	This study
AATUB1	5'-CAAATTCGTGCCCCGTGCCG	78 to 97 bp at the <i>A. alternata</i> tubulin gene (accession no. Y17073) used for RT-PCR	This study
AATUB2	5'-CTGGTAACAACCTGGGCAAG	197 to 216 bp at the <i>A. alternata</i> tubulin gene used for RT-PCR	This study
AATUB3	5'-ACTGGTCAACGACACGCTTG	1,060 to 1,041 bp at the <i>A. alternata</i> tubulin gene used for RT-PCR	This study
TP1	5'-NGTCGASWGANAWGAA	Arbitrary degenerate (AD) primer for TAIL-PCR	Liu 1995
N1	5'-TGYTGYWSNCARTTYGG	AD primer for TAIL-PCR	Arie et al. 1998

^a H = A, C, and T; K = G and T; M = A and C; N = A, C, G, and T; R = A and G; S = G and C; W = A and T; Y = C and T.

bridized when probed with an approximately 100-bp fragment at the right *EcoRV* end of the approximately 5.2-kb *Bam*HI–*EcoRV* fragment (Fig. 1A.2), suggesting that the *Bam*HI–*EcoRV* fragment included DNA common to *MAT1-1* and *MAT1-2* at the *EcoRV* end. However, when the approximately 100-bp fragment at the left *Bam*HI end of the *Bam*HI–*EcoRV*

fragment was used as a probe, it hybridized to the SUF959 DNA but not to the 880621a-1 DNA, showing that the *MAT1-2* idiomorph was truncated at the *Bam*HI (left) side of the *Bam*HI–*EcoRV* fragment and did not include flanking DNA.

TAIL-PCR was performed to obtain the rest of the *MAT1-2* idiomorph as well as the “left” flanking region (Fig. 1A.3).

A. *Fusarium oxysporum*

1. Amplification of HMG-box motif

2. Inverse-PCR

3. TAIL-PCR

4. Combined sequence

5. *MAT* genes

B. *Alternaria alternata*

1. Amplification of HMG-box motif

2. TAIL-PCR

3. Combined sequence

4. *MAT* genes

Fig. 1. Strategies for cloning the mating-type idiomorphs and structure of the loci. The base pair numbers follow the deposited sequence data in GenBank. **A, *Fusarium oxysporum*.** 1, Amplification of an approximately 190-bp fragment containing the high mobility group (HMG) box motif from strain SUF959 with primers FHM11 and FHM12. 2, Inverse- polymerase chain reaction (PCR) with *EcoRV*- and *Bam*HI-digested genomic DNA of SUF959 and primers FHM101–FHM104 identified an approximately 5.2-kb fragment containing the HMG-box motif, the “right” end of the *MAT1-2* idiomorph, and right flanking DNA of the idiomorph, common to *MAT1-1* and *MAT1-2*. 3, TAIL-PCR with SUF959 genomic DNA identified an approximately 1.9-kb fragment carrying the left end of the *MAT1-2* idiomorph and the 5’ flanking DNA common to *MAT1-1* and *MAT1-2*. 4, Combination of the sequences yielded an approximately 6.4-kb fragment that included the *MAT1-2* idiomorph and flanking regions. 5, Primers Fo25 and Fo14, designed to match the flanking regions, amplified the *MAT1-1* idiomorph (approximately 4.6 kb) with the flanking regions from strain 880621a-1 (accession no. AB0011379) and *MAT1-2* idiomorph (approximately 3.8 kb) with the flanking regions from strain SUF959 (accession no. AB0011378). **B, *Alternaria alternata*.** 1, Amplification of an approximately 270-bp fragment containing the HMG box motif from strain O-276 with primers ChHMG1 and ChHMG2 (Arie et al. 1997). 2, TAIL-PCR with O-276 genomic DNA yielded approximately 2.5- and 1.8-kb fragments that included the *MAT1-2* idiomorph and the left and right flanking regions, respectively. 3, Combination of the sequences resulted in an approximately 4.1-kb fragment that included the *MAT1-2* idiomorph and flanking regions. 4, Primers AaFS3 and AaM1-6, designed to match the flanking regions of the *MAT* idiomorphs, amplified the *MAT1-1* idiomorph (approximately 1.9 kb) with both flanking regions from strain no. 15A (accession no. AB009453) and the *MAT1-2* idiomorph (approximately 2.2 kb) with the flanking regions from strain O-276 (accession no. AB009452).

With the use of genomic DNA of SUF959 as a template, primer pair N1-Fo52 (Table 1) was used in a primary TAIL-PCR reaction, followed by a second, nested TAIL-PCR reaction with primer pair N1-Fo50 (Table 1), and an approximately 1.9-kb fragment was obtained. In DNA gel blot analysis, the fragment hybridized to genomic DNA of SUF959 and 880621a-1, suggesting that the complete *MAT1-2* idiomorph and flanking regions had been obtained. Sequence analysis of

the approximately 1.9-kb TAIL-PCR product revealed that it was actually a result of amplification with primer Fo50 only (Fig. 1A.3). These sequence data were combined with those of the *Bam*HI-*Eco*RV fragment, yielding an approximately 6.4-kb sequence that contained the *MAT1-2* idiomorph and flanking regions of SUF959 (Fig. 1A.4).

Primers Fo25 and Fo14 were designed to the 5' and 3' flanking regions of the *MAT1-2* idiomorph (Table 1 and Fig.

Fig. 2. Comparison of the *MAT1-1* alpha box (A) and *MAT1-2* high mobility group box (B) domains of fungal MAT proteins. The amino acid sequence alignments are Fo = *Fusarium oxysporum* (accession nos. AB011379 and AB011378); Nc = *Neurospora crassa* (accession nos. M33876 and M54787); Pa = *Podospora anserina* (accession nos. X64194 and X64195); Pb = *Pyrenopeziza brassicae* (accession nos. AJ006072 and AJ006073); Aa = *Alternaria alternata* (accession nos. AB009451 and AB009452); Ch = *Cochliobolus heterostrophus* (accession nos. AF029913 and AF027687); Bs = *Bipolaris sacchari* (accession no. X95814). Black-boxed residues are identical, whereas shaded boxes are similar among the sequences. Dots = gaps; arrow = conserved position of introns.

Fig. 3. Maximum likelihood tree generated by *MAT1-2* high mobility group box. *Fusarium oxysporum*, *MAT1-2* (accession no. AB011378); *Gibberella fujikuroi* (anamorph, *F. moniliforme*), *MAT1-2* (accession no. AB005041); *Nectria haematococca* (anamorph, *F. solani*), *MAT1-2* (Arie et al. 1997); *Neurospora crassa*, *mata-1* (accession no. M54787); *Cryphonectria parasitica*, *MAT1-2* (Arie et al. 1997); *Podospora anserina*, *FPR1* (accession no. X64195); *Pyrenopeziza brassicae*, *PHB1* (accession no. AJ006073); *Alternaria alternata*, *MAT1-2* (accession no. AB009452); *Cochliobolus victoriae*, *MAT1-2* (accession no. AF032369); *Bipolaris sacchari*, *MAT1-2* (accession no. X95814); *C. heterostrophus* (anamorph, *B. maydis*), *MAT1-2* (accession no. AF027687). An unrooted tree was constructed by the neighbor-joining method. The number above each horizontal line indicates the frequency with which a given branch appeared in 1,000 bootstrap trials. The bar shows a distance corresponding to five changes per 100 nucleotide positions.

1A.4) and used in long-range PCR with genomic DNA of 880621a-1 as a template, from which a 5,220-bp fragment was obtained (Fig. 1A.5). This fragment was cloned into the pCR2.1 vector and sequenced and contained a putative open reading frame (ORF) that encoded a protein with an alpha box motif (Turgeon 1998). The same primer set was also useful to amplify the 4,452-bp fragment that contained the *MAT1-2* idiomorph from SUF959.

Cloning mating-type idiomorphs of *A. alternata* by a PCR-based approach.

An approximately 270-bp fragment of HMG box motif, amplified with primers ChHMG1 and ChHMG2 (Table 1) from the genomic DNA of *A. alternata* strain O-276, were cloned and sequenced (Fig. 1B.1) (Arie et al. 1997). Strain no. 15A showed no amplification with these primers. TAIL-PCR was performed to obtain the entire *MAT1-2* idiomorph and flanking regions (Fig. 1B.2) from O-276. With the use of genomic O-276 DNA as a template, primer pairs ChHMG1-N1 (for the right side) and ChHMG2-TP1 (for the left side)

were used in the primary TAIL-PCR reaction. The second nested TAIL-PCR reaction was performed with primer pairs AaHMG3-N1 and AaHMG4-TP1 (Table 1). Approximately 2.5- and 1.8-kb fragments were obtained, which corresponded to the left and right sides of the HMG box motif, respectively. These fragments were sequenced and combined, resulting in 4,126 bp of sequence that contained the *MAT1-2* idiomorph and both flanking regions (Fig. 1B.3).

Primers AaFS3 and AaM1-6, corresponding to the 5' and 3' flanking regions of the *MAT1-2* idiomorph, respectively, were designed (Fig. 1B.3 and Table 1). A 2,690-bp fragment was obtained by PCR from the genomic DNA of strain no. 15A and then cloned and sequenced (Fig. 1B.4). The fragment contained an ORF encoding protein with an alpha box motif (Turgeon 1998). The same primer set was used to amplify a 3,001-bp fragment containing the *MAT1-2* idiomorph from O-276. The fragment was also cloned into the pCR2.1 vector.

Structural organization of *F. oxysporum* and *A. alternata* MAT idiomorphs.

Comparison of the two DNA sequences from *F. oxysporum* confirmed that the entire *MAT1-1* (4,618 bp) and *MAT1-2* (3,850 bp) idiomorphs plus flanking regions on both sides were obtained. The *MAT1-1* (880621a-1 = accession no. AB011379) and *MAT1-2* (SUF959 = accession no. AB011378) sequences have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The *MAT1-1* idiomorph carries three genes (S.-H. Yun et al. in press), one of which encodes a protein with an alpha box motif *FoMAT1-1-1* (1,194 bp), whereas the *MAT1-2* idiomorph encodes the single gene *FoMAT1-2-1* (772 bp). The *FoMAT1-1-1* ORF has an intron (48 bp), and the *FoMAT1-2-1* ORF has two introns (56 and 47 bp).

Sequence analysis of the two DNA fragments amplified with primers AaFS3 and AaM1-6 (Table 1) from *A. alternata* no. 15A and O-276 revealed that the entire *MAT1-1* idiomorph (1,906 bp) encoding a protein with an alpha-box motif and the *MAT1-2* idiomorph (2,220 bp) encoding a protein with an HMG box motif plus flanking regions on both sides were obtained. *AaMAT1-1-1* ORF (1,214 bp) has one intron (47 bp) and *AaMAT1-2-1* ORF (1,080 bp) has one intron (54 bp). Sequences of the *A. alternata* *MAT1-1* (no. 15A) and *MAT1-2* (O-276) idiomorphs with flanking regions have been deposited (*MAT1-1*, accession no. AB009451; *MAT1-2*, accession no. AB009452).

Reverse transcriptase-PCR (RT-PCR) and sequencing of PCR products confirmed that all putative introns were spliced out in the *MAT* genes of both species (data not shown). In the *MAT* genes of Ascomycetes, conserved introns were found in the HMG box and alpha box motifs (Turgeon 1998). These introns were conserved in the *MAT1-1* and *MAT1-2* of both species (Fig. 2).

AaMAT1-1-1 and *FoMAT1-1-1* are similar to *MAT* genes of the loculoascomycete *C. heterostrophus* *MAT1-1* (60.9 and 33.6% in amino acid level, respectively) and to the pyrenomyces *N. crassa* *mat A-1* (28.0 and 30.0%) and *P. anserina* *FMR1* (31.0 and 34.1%). *AaMAT1-2-1* and *FoMAT1-2-1* were similar to *C. heterostrophus* *MAT1-2* (65.3 and 26.2%, respectively), *N. crassa* *mat a-1* (29.3 and 23.3%), and *P. anserina* *FPR1* (31.9 and 22.3%) (Coppin et al. 1997; Debuchy and Coppin 1992; Glass and Staben 1997; Glass et al. 1990;

Fig. 4. Expression of the mating-type genes in *Alternaria alternata*. G1, Genomic DNA of strain no. 15A (*MAT1-1*) as the template. G2, Genomic DNA of strain O-276 (*MAT1-2*). M1, Total RNA of no. 15A grown on minimal medium (MM). M2, Total RNA of O-276 grown on MM. + = RT reaction with reverse transcriptase; - = without reverse transcriptase. *MAT1-1-1*, polymerase chain reaction (PCR) with primers AAM1-2 and AAM1-3. *MAT1-2-1*, PCR reaction with primers AAM2-2 and AAM2-3. β -tubulin, PCR reaction with primers AATUB2 and AATUB3 (as a control reaction).

Turgeon et al. 1993). The amino acid alignments of the conserved motifs are shown in Figure 2.

A phylogenetic tree was prepared based on the 71 amino acid residues of HMG box proteins and shows the similarity of the *MAT1-2* HMG box sequences (Fig. 3), which is consistent with the molecular view outlined by Alexopoulos et al. (1996).

In both asexual species, hybridization of the *MAT1-1* or *MAT1-2* idiomorph-specific probes to the genomic DNA showed that both mating types were found and that a single strain carries one or the other mating type (data not shown). This finding suggests that *F. oxysporum* and *A. alternata* are potentially heterothallic.

Transcription of *MAT* genes in *A. alternata*.

The expression of *MAT* genes was examined in *A. alternata* strains 15A (*MAT1-1*) and O-276 (*MAT1-2*) by RT-PCR. The total RNA was prepared from mycelia grown on minimal medium (MM). The first RT-PCR reaction was performed with sense primers AAM1-1, AAM2-1, and AATUB1 (Table 1) and used for amplification of *MAT1-1-1*, *MAT1-2-1*, and a β -tubulin encoding gene cDNA from *A. alternata*. For the second PCR, primer pairs AAM1-2–AAM1-3, AAM2-2–AAM2-3, and AATUB2–AATUB3 (Table 1) were used for amplification of *MAT1-1-1*, *MAT1-2-1*, and a β -tubulin encoding gene cDNA. *MAT1-1-1* and *MAT1-2-1* cDNAs were detected from strains no. 15A and O-276, respectively (Fig. 4). The cDNA of the β -tubulin encoding gene, which was thought to express constitutively, was detected from both strains. The sizes of the amplified DNA fragments correspond to the expected cDNAs. These results show that both *MAT* genes are expressed in the asexual *A. alternata*.

Heterologous expression of *A. alternata MAT* genes in *C. heterostrophus*.

Heterologous expression was performed to determine whether the *A. alternata MAT* genes are functional. *MAT1-1* (approximately 2.7 kb) and *MAT1-2* (approximately 3.0 kb) fragments from *A. alternata* no. 15A and O-276, respectively, were obtained by PCR with primers AaFS3 and AaM1-6, cloned into pCR2.1, and then cut out with *EcoRI*. *EcoRI*-digested fragments were cloned in the *EcoRI* site of pBG and designated pAaMAT1-1 and pAaMAT1-2 (Fig. 5). The resulting plasmids carrying *MAT* were transformed into *C. heterostrophus MAT* null (*MAT-0*) strain C4-41.7 (Wirsel et al. 1996), selecting for bialaphos resistance. The Bar^R transformants (ChMAT-0{pAaMAT1-1}, ChMAT-0{pAaMAT1-2}, and ChMAT-0{pBG}) were crossed with *C. heterostrophus* tester strains C5 (ChMAT1-1) and C4 (ChMAT1-2). ChMAT-0{pAaMAT1-1} $\times$ ChMAT1-2 and ChMAT-0{pAaMAT1-2} $\times$ ChMAT1-1 produced pseudothecia that were indistinguishable from those produced in wild-type matings (ChMAT1-1 $\times$ ChMAT1-2); however, asci and ascospores were less abundant (Table 2). ChMAT-0{pAaMAT1-1} $\times$ ChMAT-0{pAaMAT1-2} matings also produced pseudothecia that were indistinguishable from those produced in wild-type matings but fewer than wild-type matings. Moreover, no asci and ascospores were produced (Table 2). Control matings ChMAT-0{pAaMAT1-1} $\times$ ChMAT1-1 and ChMAT-0{pAaMAT1-2} $\times$ ChMAT1-2 produced no pseudothecium.

Mating-type assessment

of *F. oxysporum* field isolates by PCR.

Mating type was determined in field isolates of *F. oxysporum* by PCR with primer sets Falpha1–Falpha2 and FHMG11–FHMG12 (Table 1). Isolates from which an approximately 370-bp fragment was amplified with Falpha1–Falpha2 were designated *MAT1-1*, and those from which an approximately 260-bp fragment was amplified with FHMG11–FHMG12 were designated *MAT1-2* (Table 3). No strain failed to show a band with both sets of primers, and no strain showed both amplifications. All the tomato wilt pathogenic strains (*F. oxysporum* f. sp. *lycopersici*), except strain MN-60, were *MAT1-1* (Table 3). Kistler strain MN-60, the only representative of race3, was *MAT1-2*.

Mating-type assessment

of *A. alternata* field isolates by PCR.

Mating type in field isolates of *A. alternata* was determined with *MAT1-1* specific primers AAM1-1 and AAM1-2 and *MAT1-2* specific primers ChHMG1 and ChHMG2 (Table 1). Isolates from which an approximately 690-bp fragment was amplified with AAM1-1 and AAM1-2 were typed as *MAT1-1*. Those from which an approximately 270-bp fragment was amplified with ChHMG1 and ChHMG2 were typed as *MAT1-2* (Table 4), which confirmed the earlier findings (Arie et al. 1997). Each strain had one or the other of the *MAT* genes. We could not find any relationship between the pathotypes and mating types.

DISCUSSION

Evolutionary theory indicates that sex plays important roles in pathogen evolution, i.e., the development of a new pathogenic race compatible to a disease-resistant cultivar and of a new strain resistant to a fungicide. However, no sexual stage is known for more than 15,000 species of fungi, and many phytopathogenic species such as *F. oxysporum* and *A. alternata* are asexual.

Fig. 5. Plasmids pAaMAT1-1 and pAaMAT1-2 used for transformation of *Cochliobolus heterostrophus* C4-41.7 (*MAT-0*). Open boxes indicate *MAT* idiomorphs; solid boxes indicate flanking DNA. The *Alternaria alternata MAT1-1-1* and *MAT1-2-1* open reading frames are shown as arrows in boxes. The arrows at the left and right sides of the *MAT* idiomorphs indicate primers used for polymerase chain reaction. *amp* = *Escherichia coli* ampicillin resistance gene; *ori* = *E. coli* origin of replication; *GPD1* = *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene promoter; *bar* = *E. coli* bialaphos resistance gene.

Our interest is to clarify the mechanisms of asexuality in asexual ascomycetes in molecular viewpoints and to elucidate the cause of loss of sexual reproduction. To achieve these goals, analysis of mating mechanisms in ascomycetes is essential. Recently, *MAT* loci have been isolated from several species of filamentous ascomycetes. The loci appeared to

function as regulators of complex genetic pathways containing numerous genes (Leslie and Klein 1996; Turgeon 1998).

In this report, the two asexual ascomycetes *F. oxysporum* and *A. alternata* were proved to have *MAT* loci, which are structurally similar to those of a heterothallic species. *MAT* genes are expressed in *A. alternata* where *MAT1-1* as well as

Table 2. Expression of the *Alternaria alternata* *MAT1-1* and *MAT1-2* in *Cochliobolus heterostrophus* strain C4-41.7 (*MAT*-0)

Cross ^a	Pseudothecium formation	Ascospore production
ChMAT1-1 × ChMAT1-2	+	+
ChMAT1-1 × ChMAT-0	—	—
ChMAT1-2 × ChMAT-0	—	—
ChMAT1-1 × ChMAT-0{pBG}	—	—
ChMAT1-2 × ChMAT-0{pBG}	—	—
ChMAT1-1 × ChMAT-0{pAaMAT1-1}	—	—
ChMAT1-1 × ChMAT-0{pAaMAT1-2}	+ ^b	+ ^c
ChMAT1-2 × ChMAT-0{pAaMAT1-1}	+ ^b	+ ^c
ChMAT1-2 × ChMAT-0{pAaMAT1-2}	—	—
ChMAT-0{pAaMAT1-1} × ChMAT-0{pAaMAT1-2}	+ ^d	—

^a ChMAT1-1 = *C. heterostrophus* strain C5 (*MAT*1-1); ChMAT1-2 = strain C4 (*MAT*1-2); ChMAT-0 = strain C4-41.7, a *MAT* deletion mutant (Wirsal et al. 1996); ChMAT-0{pAaMAT1-1} = transformants derived by transformation of strain C4-41.7 with pAaMAT1-1; ChMAT-0{pAaMAT1-2} = transformants derived from strain C4-41.7 with pAaMAT1-2; ChMAT-0{pBG} = transformants derived from strain C4-41.7 with pBG.

^b Pseudothecia formed were indistinguishable from those produced in wild-type matings (ChMAT1-1 × ChMAT1-2), but the number was smaller than the wild-type matings.

^c Only a few asci and ascospores were produced.

^d Pseudothecia, which were indistinguishable from those produced in wild-type matings, were formed.

Table 3. Mating-type determination of *Fusarium oxysporum* field isolates by polymerase chain reaction (PCR)

Forma specialis (host), race, and isolate number	Source	Origin	PCR product with Falpha1–Falpha2 ^a	PCR product with FHMGI1–FHMGI2 ^b	Mating type
<i>f. sp. lycopersici</i> (tomato)					
race1					
6531	Institute for Fermentation (IFO), Osaka	Kyushu, Japan	+	—	<i>MAT</i> 1-1
16417	American Type Culture Collection (ATCC)	U.S.A	+	—	<i>MAT</i> 1-1
103036	National Institute of Agro-Environmental Science (MAFF)	Mie, Japan	+	—	<i>MAT</i> 1-1
103038	MAFF	Ibaraki, Japan	+	—	<i>MAT</i> 1-1
103042	MAFF	Utsunomiya, Japan	+	—	<i>MAT</i> 1-1
103043	MAFF	Ichikai, Japan	+	—	<i>MAT</i> 1-1
305121	MAFF	Japan	+	—	<i>MAT</i> 1-1
305559	MAFF	Shizuoka, Japan	+	—	<i>MAT</i> 1-1
727501	MAFF	Japan	+	—	<i>MAT</i> 1-1
744006	MAFF	Fukuoka, Japan	+	—	<i>MAT</i> 1-1
race2					
880621a-1	T. Arie	Imaichi, Japan	+	—	<i>MAT</i> 1-1
1330	M. Shimosaka (SUF)	Japan	+	—	<i>MAT</i> 1-1
16605	ATCC	U.S.A.	+	—	<i>MAT</i> 1-1
46933	ATCC	South Africa	+	—	<i>MAT</i> 1-1
race3					
MN-60	H. C. Kistler	FL, U.S.A.	—	+	<i>MAT</i> 1-2
<i>f. sp. radialis-lycopersici</i> (tomato)					
103044	MAFF	Gifu, Japan	+	—	<i>MAT</i> 1-1
959	SUF	Japan	—	+	<i>MAT</i> 1-2
103047	MAFF	Kochi, Japan	—	+	<i>MAT</i> 1-2
<i>f. sp. melongenae</i> (egg plant)					
103051	MAFF	Ibaraki, Japan	+	—	<i>MAT</i> 1-1
<i>f. sp. conglutinans</i> (cabbage)					
1-1	Y. Nomura	Tokyo, Japan	+	—	<i>MAT</i> 1-1
<i>f. sp. batatas</i> (sweet potato)					
O-17	K. Watanabe	Ibaraki, Japan	—	+	<i>MAT</i> 1-2
<i>f. sp. apii</i> (celery)					
1017	M. Shimosaka	Chiba, Japan	—	+	<i>MAT</i> 1-2

^a + = approximately 370 bp of product; — = no product.

^b + = approximately 190 bp of product; — = no product.

MAT1-2 are functional. These results were similar to those reported in *B. sacchari* (Sharon et al. 1996). Two possible reasons for asexuality, loss of *MAT* genes and functional disruption of one or both *MAT* genes, have been refuted for *A. alternata* as well as for *B. sacchari* (Sharon et al. 1996). Thus, asexuality must be attributed to something other than *MAT*. This may be a result of the functional disorder of the yet unidentified genes that are involved in successful sexual reproduction.

Leslie and Klein (1996) discussed in their paper that the idealized fungal individual is a haploid, self-sterile, hermaphrodite (heterothallic). Normally, female sterile mutants derived from a heterothallic strain are at a selective disadvantage in sexual reproduction. Female sterile mutants must have the advantage during vegetative propagation in order to persist at a significant frequency, thus allowing female sterile strains to accumulate to a level such that local populations completely lose sexuality and appear as an asexual species. However, Taylor et al. (1999) suggested that all asexual fungi are originally heterothallic and most of them should be potentially heterothallic (not asexual).

Our data on mating-type assessment of field isolates show that all strains of *F. oxysporum* f. sp. *lycopersici* (pathogen of tomato wilt), except Kistler strain MN-60 (race 3) that should be from a different lineage (O'Donnell et al. 1998), carried *MAT1-1* (Table 3). This suggests two possible reasons for asexuality in *F. oxysporum* f. sp. *lycopersici*: the fungus is a female sterile mutant from the original heterothallic fungus and has become a collection of asexual clones and, although the fungus still has the ability to reproduce sexually, no mating partner could be found in the population at this moment. Taylor et al. (1999) mentioned that the factors categorized into biology, space, and time affect differentiation of heterothallic and asexual manners of fungi and that host preference (segregation of formae speciales) is one of the important biotic factors. Further studies on the evolution of fungal pathogenicity will reveal the mechanism by which they are asexual and, moreover, whether they are truly asexual or not.

MATERIALS AND METHODS

Strains, culture conditions, and crosses.

F. oxysporum f. sp. *radicis-lycopersici* strain SUF959 and f. sp. *lycopersici* strain 880621a-1 (this laboratory) and Japanese

pear pathotype of *A. alternata* strains O-276 and no. 15A were used to clone the *MAT* genes. *C. heterostrophus* strains C5, C4, and C4-41.7 were obtained from O. C. Yoder and B. G. Turgeon of Cornell University (Ithaca, NY, U.S.A.). Strain C4-41.7 is a *MAT-0* strain derived from C4 that lacks the whole mating-type locus (Wirsel et al. 1996). Field isolates used for assessment of mating type are described in Tables 3 and 4. Strains originated from foreign countries were imported under permits from the Ministry of Agriculture, Forestry and Fisheries of Japan. Media, growth, storage, and cross conditions have been described (Arie et al. 1998; Leach et al. 1982; Sharon et al. 1996; Wirsel et al. 1996).

DNA and RNA manipulations.

Genomic DNAs of *F. oxysporum* and *A. alternata* were purified as described (Arie et al. 1998). DNA ligation for inverse-PCR was performed with a TaKaRa DNA Ligation Kit, version 2 (Takara, Kusatsu, Japan), at 16°C for 12 h.

For DNA blot analysis, approximately 1 µg of restriction enzyme-digested genomic DNA was separated in a 0.8% (wt/vol) agarose gel. Gel blots on positively charged polyamide membrane (Nytran-plus, Schleicher & Schuell, Keene, NH, U.S.A.) were prepared by alkaline transblotting and hybridized with probes prepared with a DIG DNA Labeling and Detection Kit Nonradioactive (Boehringer Mannheim GmbH, Boehringer, Germany), according to the manufacturer's instructions.

For RNA extraction, mycelia of *A. alternata* harvested from 100 ml of MM (Leach et al. 1982) in 200-ml Erlenmeyer flasks and cultured (120 rpm) at 25°C for approximately 3 days were ground in liquid nitrogen with a pestle in mortar. Total RNA was prepared from the powdered mycelia as described by Timberlake (1986).

PCR.

Primers used for PCR are listed in Table 1. All the primers were synthesized by Boehringer Mannheim Nihon Gene Research Laboratory (Sendai, Japan), dissolved (100 µM) in water, and stored at -20°C.

Each PCR reaction mixture (final volume 50 µl) contained 1× PCR Buffer II (PE Applied Biosystems, Foster City, CA, U.S.A.), 2.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate (dNTP), 2 µM of each primer, 0.025 U AmpliTaq Gold (PE Applied Biosystems), and about 20 ng of fungal genomic

Table 4. Mating type determination of *Alternaria alternata* field isolates by polymerase chain reaction (PCR)

Pathotype and isolate number	Geographic origin	PCR product with AAM1-1 and AAM1-2 ^a	PCR product with ChHMG1 and ChHMG2 ^b	Mating type
Japanese pear				
No. 15A	Tottori, Japan	+	—	MAT1-1
O-276	Tottori, Japan	—	+	MAT1-2
Apple				
M-71	Toyama, Japan	+	—	MAT1-1
O-159	Nagano, Japan	+	—	MAT1-1
Tomato				
O-228	Japan	+	—	MAT1-1
As-27	Japan	+	—	MAT1-1
Saprophyte (nonpathogenic)				
IMI89843	U.K.	—	+	MAT1-2
EGS35-913	U.S.A.	—	+	MAT1-2
O-94	Tottori, Japan	+	—	MAT1-1

^a + = approximately 690 bp of product; — = no product.

^b + = approximately 270 bp of product; — = no product. Data from Arie et al. 1997.

DNA. Thermal conditions were denaturation at 95°C for 15 min; 35 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min; final extension at 72°C for 10 min.

To obtain a long PCR fragment (long-range PCR), reaction mixtures (final volume 50 µl) contained 1× LA PCR Buffer (Takara), 0.4 mM dNTP, 1 µM of each primer, 0.5 µl of TaKaRa LA Taq (Takara), and about 20 ng of template DNA. Cycling conditions were denaturation at 94°C for 1 min; 30 cycles at 98°C for 20 s and 58°C for 10 min; final extension at 72°C for 10 min.

Primary TAIL-PCR reaction mixtures (final volume 25 µl) contained 1× PCR Buffer II, 2.5 mM MgCl₂, 0.2 mM dNTP, 0.4 µM specific primer, 5 µM arbitrary degenerate (AD) primer, 0.016 U of AmpliTaq, and approximately 20 ng of genomic DNA. The secondary amplification reactions (final volume 50 µl) contained the same components as the primary reactions with the addition of 0.2 µM nested specific primer, 4 µM AD primer (same as used in the primary reaction), and 0.012 U of AmpliTaq. The template was 1 µl of a 1:50 dilution of the primary reaction mixture. Thermal conditions for TAIL-PCR were described by Liu and Whittier (1995).

RT-PCR was performed with a TaKaRa RNA PCR Kit Avian Myeloblastosis Virus (AMV), version 2.1 (Takara). The RT reaction mixture (20 µl), containing 1 µg of total RNA, 0.125 µM Oligo dT-Adapter Primer, 1 mM dNTP (each), and 5 U of AMV reverse transcriptase XL, was incubated at 42°C for 1 h and the enzyme was inactivated at 99°C for 5 min. For the first PCR reaction, the whole mixture was added with 20 pmol of each M13 primer M4, the appropriate sense primer, and 2.5 U of TaKaRa Taq. The first PCR was performed for 30 cycles with an initial 2 min at 94°C. Each cycle consisted of 30 s at 94°C, followed by 30 s at 55°C and 2 min at 72°C. To exclude nonspecific products, the second PCR reaction was performed with the appropriate nested primers. The second PCR reaction mixture, in a total volume of 50 µl, contained 1 µl of the first PCR reaction mixture, 1 µM nested primer (each), 200 µM dNTP (each), and 1.25 U of AmpliTaq Gold. The second PCR was performed for 25 cycles with an initial 10 min at 94°C and a final 10 min at 72°C. Each cycle consisted of 1 min at 94°C, followed by 30 s at 55°C and 2 min at 72°C.

Cloning, sequencing, and analysis of PCR products.

PCR products were cloned into vector pCR2.1 (Invitrogen, San Diego, CA, U.S.A.) or into pGEM-T easy vector (Promega, Madison, WI, U.S.A.), following the recommendations of the suppliers. Plasmid DNA was purified with an RPM Kit (BIO 101, Carlsbad, CA, U.S.A.), following the manufacturer's recommendation. The inserts were sequenced with the M13 reverse or M13 (-40) forward primers. DNA sequences were determined by the ALFexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) and automated fluorescent DNA sequencer 377 (PE Applied Biosystems) at the RIKEN Biodesign DNA Sequencing Facility. Sequences were assembled and analyzed with the GeneWorks (IntelliGenetics, Mountain View, CA, U.S.A.) and Genetyx-Mac (Software Development, Tokyo, Japan) programs. BLAST (Altschul et al. 1990) searches were done against the NCBI-GenBank databases.

Amino acid alignment and phylogenetic analysis.

The multiple alignments of the HMG box and alpha box were constructed with the ClustalW program (Thompson et al. 1994) and adjusted manually. An unrooted phylogenetic tree was generated by the neighbor-joining method (Saitou and Nei 1987) on 71 amino acid residues of the HMG box protein in a *MAT1-2-1* homolog for 11 ascomycetous species.

Transformation of *C. heterostrophus*.

Plasmid pBG, which carries *bar*-encoding resistance to bialaphos (Straubinger et al. 1992), was obtained from Turgeon and Yoder (Fig. 5) (Wirsal 1996). Transformation of protoplasts, derived from mycelia of *C. heterostrophus* C4-41.7, followed protocols described previously (Turgeon et al. 1993) with 2 mg of Novozyme 234 (Novo Nordisk, Bagsvaerd, Denmark) per milliliter and 10 mg of Driselase (Kyowa Hakko, Tokyo, Japan) per milliliter in 0.7 N NaCl. Bar^R transformants were selected on regeneration medium containing 100 µg of bialaphos per milliliter (Meiji Seika Kaisha, Tokyo, Japan).

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