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FULL PAPER

Molecular organization of the mating type (*Mat*) locus of *Exserohilum monoceras* (*Setosphaeria monoceras*), a bioherbicide agent for *Echinochloa* weeds

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Abstract Mating-type genes were cloned and sequenced in the heterothallic phytopathogenic fungus *Exserohilum monoceras*, a candidate bioherbicide for the control of *Echinochloa* weeds in rice fields. Using PCR-based methods, we determined both idiomorphs and flanking regions of these genes. The structural organization of the *E. monoceras Mat* locus was similar to that of other heterothallic ascomycetes. The *MAT1-1-1* gene was 1191 bp and encoded a predicted protein of 379 amino acids with an α box domain. The *MAT1-2-1* gene was 1093 bp and encoded a predicted protein of 345 amino acids with a high mobility group box domain. Expression of both *MAT* genes was detected under vegetative and invasive growth conditions. *MAT*-specific primers were developed to assess the mating-type frequency of *E. monoceras* field isolates. Both mating types were observed in Japanese field isolates. Analysis of mating types and sexual hybridization of *E. monoceras* could provide useful approaches for the conventional genetic manipulation of this fungus to produce a more efficient bioherbicide.

Keywords Barnyard grass · Mycoherbicide · Pleosporales · Population · Sexual reproduction

Introduction

Exserohilum monoceras is a phytopathogenic fungus that is found widely in Southeast Asia, North America, and Australia. The fungus causes severe leaf blight in *Echinochloa* weed species, which are considered to be the world's most harmful weed species. *Echinochloa* weed species compete with many economically important crops (e.g., banana, cassava, cotton, corn, millet, potato, sorghum, and taro) and represent a serious problem in rice production (Holm et al. 1977). However, because *E. monoceras* causes only slight damage to rice plants, it is considered a promising candidate bioherbicide in rice fields (Tsukamoto et al. 1997; Zhang and Watson 1997). Excessive applications of agrochemicals have been causing increasing concern regarding their effects and risks to the environment. In addition, consumer preferences for non-chemical herbicides and the emergence of herbicide-resistant weeds have also stimulated the development of useful phytopathogenic mycoherbicides (Charudattan 2001).

The intensity of pathogenicity, the host-specificity system, mass production of inocula, and application methods are of major importance in the development of suitable bioherbicides (Charudattan 2005). Genetic manipulation and in vitro cultivation conditions must be established to analyze these aspects of biocontrol. Biochemical and molecular genetic manipulation is a valuable tool for elucidating fungal pathogenic features. Conventional breeding strategies are essential for improving effective strains because non-genetically modified mycoherbicides should be released in the field for ecologically friendly agriculture. However, to date, there have been few studies on the molecular and conventional genetic manipulation of *E. monoceras*.

The perfect stage of *E. monoceras* (*Setosphaeria monoceras*) was discovered by Alcorn (1978) after in vitro crosses of Australian strains; these crosses revealed that the fungus heterothallic with a bipolar system. However, further crossings and observations of the sexual state of this fungus have not been demonstrated in other locations, such as the tropics or East Asia, where the application of the

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bioherbicide is expected. In *E. monoceras*, mating systems or conditions and mating-type frequency have not been examined in detail. Sexual reproduction, during which gene exchange occurs and genetic variation in the population is enriched, is a special event in the fungal life cycle (Milgroom 1996; Kronstad and Staben 1997; Turgeon 1998). Elucidation of a sexual cycle provides an invaluable tool for classical genetic analysis and offers insights into the role or systems of sexual reproduction in *E. monoceras* and related phytopathogenic fungal species.

In bipolar heterothallic ascomycetes, the mating locus consists of highly divergent DNA sequences in compatible mating-type strains, even though the sequences occupy the same chromosomal position; these alleles are known as idiomorphs (Turgeon 1998). The mating-type idiomorphs (*MAT1-1* and *MAT1-2*) contain one or more genes that allow sexual reproduction to occur. *MAT1-1* encodes an α box protein and *MAT1-2* encodes a high mobility group (HMG) protein; both contain DNA-binding motifs. These proteins are transcription factors and may regulate signal transduction cascades for sexual reproduction (Nelson 1996; Turgeon 1998; Izumitsu et al. 2009). The gradual accumulation of such molecular genetic information has revealed the presence of *MAT* genes in species that have long been considered asexual, suggesting that these species may be heterothallic (Sharon et al. 1996; Arie et al. 2000). PCR-based mating-type gene determination is accurate and rapid (Gafur et al. 1997) and has revealed the mating-type composition in specific asexual populations of sexual fungi (Bennett et al. 2003; Tredway et al. 2003). A recent striking example that such techniques have been instrumental in identifying the sexual property was the discovery of a teleomorph in *Aspergillus fumigatus* (O'Gorman et al. 2009). The sexual cycles of this clinically important fungus had remained unknown until molecular genetics provided evidence for the presence of *MAT* genes, the expression of sex-related genes, or recombinations (Pöggeler 2002; Paoletti et al. 2005; O'Gorman et al. 2009).

In the study reported here, we identified and structurally analyzed the mating-type genes and determined the molecular organization of *Mat* locus in *E. monoceras*. The expression patterns of *MAT* genes were examined. Based on the *MAT* sequences, we developed multiplex PCR primers which we used to demonstrate the mating-type frequency in Japanese *E. monoceras* populations.

Materials and methods

Strains, culture, and DNA extraction

The 35 strains of *E. monoceras* used in this study are described in detail in Table 1. All were collected from diseased *Echinochloa* weeds or *Oplismenus undulatifolius* in Japan. Laboratory strains 9.29 and IB were obtained from the Pesticide Research Institute, Faculty of Agriculture, Kyoto University. A stock culture of JTB-808 (=NBRC 100161) was a gift from Dr. Tsukamoto, Applied Plant Research Center, Japan Tobacco, Inc. For DNA extraction, mycelia were grown in a liquid complete medium (CM; Tanaka et al. 1991) for approximately 4 days. Genomic DNA was extracted from mycelia ground in liquid nitrogen and purified as described previously (Saitoh et al. 2010).

Cloning of *MAT* genes

Unless otherwise noted, PCR was performed with KOD Dash DNA polymerase (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Primers used in the amplification of the *MAT* genes are listed in Table 2. To isolate α box and HMG box sequences, we performed PCR using degenerate primers. Degenerate primers (1 + 2 and 3 + 4) were designed by aligning *MAT* gene sequences from related loculoascomycetes *Cochliobolus heterostrophus* [DDBJ/EMBL/GenBank Acc. Nos. AF027687 and AF029913), *C. sativus* (AF275373 and AF275374), *Alternaria alternata* (AB444180 and AB444173), *Leptosphaeria maculans* (AY174048 and AY174049), and *Phaeosphaeria nodorum* (AY212018 and AY212019)]. *Taq* DNA polymerase (Promega, Madison, WI) was used, and the following cycling program was adopted for the reaction: initial denaturation at 94°C for 2 min; 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 52°C, and extension for 1 min at 72°C; a final extension for 5 min at 72°C. The resultant PCR fragments were cloned into pZErO-2 vector (Invitrogen, Carlsbad, CA) and sequenced.

The flanking regions of the α box and HMG box sequences were amplified using the inverse PCR method (Ochman et al. 1988). Genomic DNA from *E. monoceras* YM-1 (*MAT1-1* strain) and 9.29 (*MAT1-2* strain) was digested using six-cutter restriction enzymes (*Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I: TAKARA Bio, Otsu, Japan). After self-ligation with T4 DNA ligase (Fermentus UAB, Vilnius, Lithuania), DNA was purified and used as a template for the PCR analyses. Enzymatic reactions were performed following the recommendations of the supplier. PCR was performed with TaKaRa LA *Taq* polymerase (TAKARA Bio) under the following cycling conditions: initial denaturation at 94°C for 3 min; 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 60°C, and extension for 3 min at 72°C; a final extension for 10 min at 72°C. Primers (5 + 6 and 7 + 8) were designed outward, based on obtained sequences. The resulting fragments were cloned and sequenced.

Table 1 Fungal isolates used in this study

Name	Host	Origin	Mating type
9.29[a]	*Echinochloa oryzicola*	Stock culture	*MAT1-2*[c]
JTB-808	*Echinochloa* spp.	Stock culture[b]	*MAT1-1*
D. kita-1	*Echinochloa crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-2*
D. kita-2	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-2*
D. kita-3	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-2*
D. kita-4	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-1*
D. delta-1[a]	*E. oryzicola*	Daigo, Fushimi, Kyoto City	*MAT1-1*[c]
D. delta-2	*E. oryzicola*	Daigo, Fushimi, Kyoto City	*MAT1-2*[c]
D. mukai-1	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-2*
D. mukai-2[a]	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-1*
D. 10.3-1	*E. crus-galli*	Daigo, Fushimi, Kyoto City	*MAT1-2*
D. dankita-2	*E. oryzicola*	Daigo, Fushimi, Kyoto City	*MAT1-2*
Kibune	*E. oryzicola*	Kibune, Sakyo, Kyoto City	*MAT1-2*[c]
Wakare	*E. oryzicola*	Kitashirakawa, Sakyo, Kyoto City	*MAT1-2*
YM-1[a]	*E. crus-galli*	Kofu City, Yamanashi Pref.	*MAT1-1*[c]
YM-2[a]	*E. crus-galli*	Kofu City, Yamanashi Pref.	*MAT1-1*[c]
Kindai-1	*E. crus-galli*	Kanazawa City, Ishikawa Pref.	*MAT1-1*
Kindai-2	*E. crus-galli*	Kanazawa City, Ishikawa Pref.	*MAT1-2*[c]
Kindai-3	*E. crus-galli*	Kanazawa City, Ishikawa Pref.	*MAT1-2*
Nishi1-1[a]	*E. oryzicola*	Kousei, Konan City, Shiga Pref.	*MAT1-1*[c]
Nishi1-2[a]	*E. oryzicola*	Kousei, Konan City, Shiga Pref.	*MAT1-1*
Nishi1-3[a]	*E. oryzicola*	Kousei, Konan City, Shiga Pref.	*MAT1-1*
IB	*E. oryzicola*	Stock culture	*MAT1-2*
C. ex-1[a]	*E. colona*	Iwakura, Sakyo, Kyoto City	*MAT1-1*[c]
C. ex-2	*E. colona*	Iwakura, Sakyo, Kyoto City	*MAT1-2*[c]
C. ex-3	*E. colona*	Iwakura, Sakyo, Kyoto City	*MAT1-2*
C. ex-4	*E. colona*	Iwakura, Sakyo, Kyoto City	*MAT1-2*
C. ex-5	*E. colona*	Iwakura, Sakyo, Kyoto City	*MAT1-2*
T. uji-1	*Oplismenus undulatifolius*	Uji City, Kyoto Pref.	*MAT1-2*
T. uji-2	*O. undulatifolius*	Uji City, Kyoto Pref.	*MAT1-2*
T. uji-3	*O. undulatifolius*	Uji City, Kyoto Pref.	*MAT1-2*
T. uji-4	*O. undulatifolius*	Uji City, Kyoto Pref.	*MAT1-2*
T. maruyama-1	*O. undulatifolius*	Higashiyama, Kyoto City	*MAT1-2*[c]
T. maruyama-2	*O. undulatifolius*	Higashiyama, Kyoto City	*MAT1-1*[c]
T. petori	*O. undulatifolius*	Stock culture	*MAT1-2*

Mating types were determined by the multiplex PCR method

[a] Vouchers were deposited at CBM (FB-38673–FB-38681)

[b] =NBRC100161 (Tsukamoto et al. 1997)

[c] The results of multiplex PCR are shown in Fig. 6

Sequence analysis and alignment of *MAT* genes

The sequencing reaction was performed with a Dye Terminator CEQ-DTCS Quick Start kit (Beckman Coulter, Brea, CA), and the nucleotide sequence was determined using the CEQ 2000 DNA Analysis System sequencer (Beckman Coulter), as described previously (Yoshimi et al. 2004). The sequences were aligned and the amino acid sequences predicted using DNAStar SeqMan program (DNAStar, Madison, WI). Homology searches were performed with BLASTX (Altschul et al. 1997). Protein sequences were aligned using ClustalW (Thompson et al. 1994; http://www.ebi.ac.uk/clustalw), and protein domain prediction was performed using InterProScan (Zdobnov and Apweiler 2001; http://www.ebi.ac.uk/InterProScan).

Transcripts detection by reverse transcriptase-PCR

RNA for the expression analysis of *MAT* genes was prepared from mycelia grown first in liquid CM, with shaking at 25°C for 4 days, then in liquid minimal medium (MM; CM without yeast extracts and tryptone), with shaking at 25°C for 4 days, followed by inoculation into the host plant. Inoculation was performed as follows: a 2 × 2-mm fungal culture plug was placed on the leaf blades of 4-week-old *E. oryzicola* with a drop of 0.02% Tween 20.

Table 2 Sequences of primers used to amplify fragments of the *E. monoceras MAT* idiomorphs

Primer number	Name	Sequence 5′–3′	Amplicon size (bp)	
			MAT1-1	*MAT1-2*
1	M1degefr	AGGCTCTYAATGCSTTCGTYGGATTC	620	
2	M1degerv	GCTTRGCTCGGCGYTTRTTRCGTG		
3	M2degefr	TGAACTGYTGGATCATYTTSCGAG		254
4	M2degerv	CTGTACTTGTAGTYGGSGTGCTG		
5	M1invfr	ACACCAACACCACCTCACAGAC		
6	M1invrv	GTGCTTGAAGGTAGGGATTGTG		
7	M2invfr	CTCGTATATGGCACAACCTGTCTC		
8	M2invrv	GAATTCTGCATTAAGATGTTTGTG		
9	M1rtfr	AGAGACCCAACCGGTGCTG	305	
10	M1rtrv	AGTTTCTTCATAGGCCAGTGCTTG	(cDNA 254)	
11	M2rtfr	TGAACTGCTGGATCATTTTCCGAG	372	
12	M2rtrv	TGACTTTGTGAGGTGAGGTGAC	(cDNA 317)	
13	ORF1rtfr	GGTGCACGACTGGATATCTCATC	343	343
14	ORF1rtrv	GCATTGGTTTCCTGTGTCACCTTC	(cDNA 343)	(cDNA 343)
15	MultiM1fr	TCACAGACCTTCCTCGGACAGTTG	684	
16	MultiM2fr	CCCTCAAAATCTTTCGACTC		405
17	Multicommonrv	GGTGGCTGCATCGAGCAAACACAT		

fr forward, *rv* reverse

Inoculated plants were kept at 25°C and 100% relative humidity in a plastic chamber for 4 days. Mycelia and the inoculated parts of the plant were ground in liquid nitrogen, and total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was prepared with the Superscript II reverse transcriptase (RT) kit (Invitrogen) and poly T primer, according to the manufacturer's recommendations. cDNA was subjected to PCR using KOD Dash polymerase and primers for genes in the *Mat* locus under the following reaction conditions: an initial denaturing step at 94°C for 2 min; 30 cycles of 94°C for 30 s, 57°C for 2 s, and 74°C for 1 min; a final extension step at 74°C for 5 min (Table 2). The *GPD* (glucose-6-phosphate dehydrogenase) gene of *E. monoceras*, of which ortholog in a fungus is constitutionally expressed during a growing state by its promoter, was used as the control for the cDNA preparations (Punt et al. 1988; Van Wert and Yoder 1992; Tanaka et al. 2011; our unpublished data). Genomic DNAs from the YM-1 and 9.29 strains were also used for PCR amplification to obtain the control PCR products from genomic *MAT* and *GPD* genes.

Multiplex PCR assay

Genomic DNA was prepared from all of the *E. monoceras* isolates listed in Table 1. *MAT1-1-1*-specific forward primer 15 (MultiM1fr) and *MAT1-2-1*-specific forward primer 16 (MultiM2fr) were designed within each idiomorph. In the 3′ flanking region of both idiomorphs, a common reverse primer 17 (Multicommonrv) was designed that amplified a 684-bp fragment with primer 15 in *MAT1-1* strains and a 405-bp fragment with primer 16 in *MAT1-2* strains, respectively. A mixture of the three primers was used at a final concentration of 1 μM for the multiplex PCR reaction (an initial denaturing step at 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; a final extension step at 72°C for 5 min) with *Taq* DNA polymerase (Promega).

Results

Cloning and sequence analysis of *E. monoceras MAT* genes

To search for *MAT* genes in the *E. monoceras* genome, we designed two degenerate primer sets (1 + 2 and 3 + 4, described in Table 2) that corresponded to the conserved motifs of the α box and HMG box of several phylogenetically related Pleosporales fungi. Using PCR, we tested each primer set against genomic DNA obtained from six field isolates of *E. monoceras*. The α box primers amplified approximately 680-bp products from two isolates, and the HMG box primers amplified approximately 260-bp products from the remaining four isolates. For further molecular analysis, we chose YM-1 and 9.29 as representative strains; both had stable growth in vitro and high virulence. Nucleotide sequences of YM-1 and 9.29 DNA products showed significant similarities to fungal *MAT1-1-1* and *MAT1-2-1* genes, respectively (data not shown).

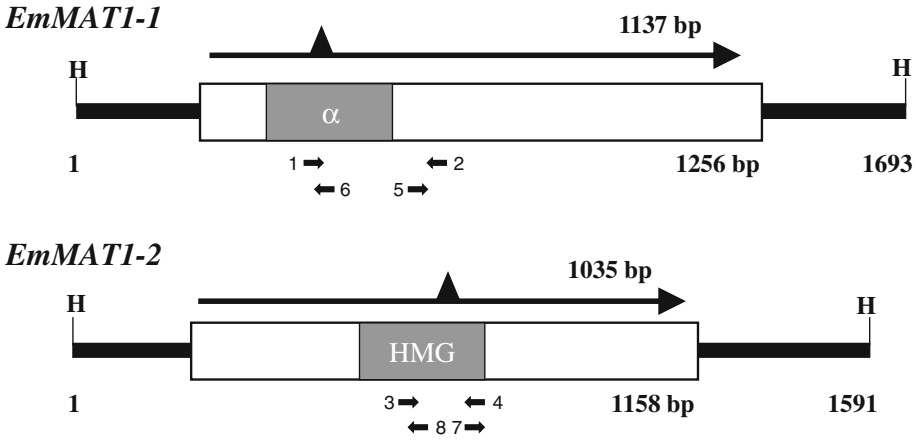

Fig. 1 Sequence organization of inverse PCR fragments of *Exserohilum monoceras MAT1-1* and *MAT1-2* strains. *White boxes* Idiomorphs, *shaded regions* α and high mobility group (HMG) boxes, respectively, *arrows above idiomorphs MAT1-1-1* and *MAT1-2-1* genes, *black triangles* intron positions, *thick black lines* common flanking regions. *H* at both ends of the fragments are *Hin*dIII sites. The positions of primers used for degenerate PCR and inverse PCR are given by *arrows below the idiomorph boxes*

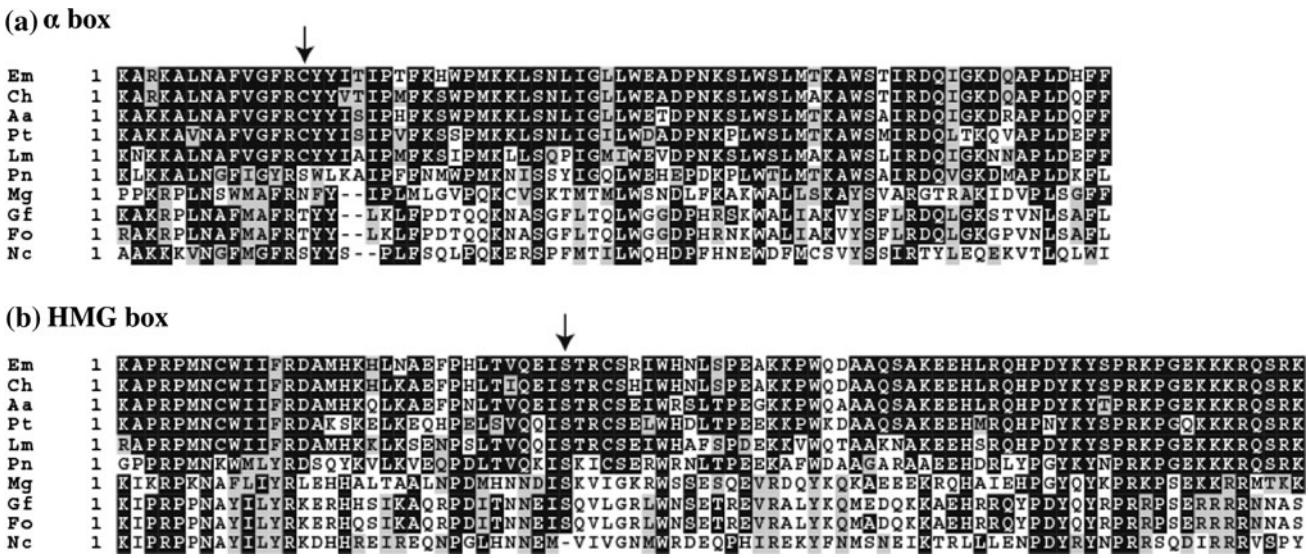

Fig. 2 Multiple alignment of deduced amino acid sequence of α box (**a**) and HMG box (**b**) regions from *E. monoceras*. *Vertical arrows* Positions of conserved introns. *Em E. monoceras*, *Ch Cochliobolus heterostrophus*, *Aa Alternaria alternata*, *Pt Pyrenophora teres*, *Lm Leptosphaeria maculans*, *Pn Phaeosphaeria nodorum*, *Mg Mycosphaerella graminicola*, *Gf Gibberella fujikuroi*, *Fo Fusarium oxysporum*, *Nc Neurospora crassa*

To obtain the entire *MAT1-1-1* gene sequence and the surrounding region from strain YM-1, inverse PCR was performed. An approximately 1.7-kbp product was amplified from the *Hin*dIII-digested DNA. Sequence analysis of the product confirmed that the amplicon contained a single *MAT1-1-1* open reading frame (ORF), which was 1137 bp in length and encoded a predicted protein of 379 amino acids (Fig. 1). The length of the ORF was comparable to known *MAT1-1-1* genes of Pleosporales fungi. The amino acid sequences of *E. monoceras MAT1-1-1* demonstrated a high similarity throughout the entire sequence to those sequences of MAT1-1-1 proteins from other heterothallic fungal species, such as *Cochliobolus heterostrophus* (70% identity and 79% similarity), *Alternaria alternata* (57% identity and 71% similarity), and *Leptosphaeria maculans* (44% identity and 57% similarity). MAT1-1-1 proteins or sequences in homothallic fungi also showed high similarities to *E. monoceras,* e.g., *Cochliobolus kusanoi* (69% identity and 79% similarity), *Pleospora tarda* (74% identity and 82% similarity), and *Cochliobolus homomorphus* (70% identity and 81% similarity). The homology was especially high throughout the α box region. A putative

intron was identified within the α box that was at the same position as in the *MAT1-1-1* genes of *C. heterostrophus*, *Pyrenophora teres*, *L. maculans*, *A. alternata*, *A. fumigatus*, and other ascomycetes (Fig. 2a). At the 3′ and 5′ ends of the 51-bp intron, typical fungal splice sites were found.

As in *MAT1-1-1*, inverse PCR amplified an approximately 1.5-kbp product from the *Hin*dIII-digested DNA of *MAT1-2* strain 9.29. Sequence analysis revealed that the amplicon contained a 1035-bp *MAT1-2-1* ORF. The nucleotide sequences of the 3′ and 5′ terminal regions of the product were identical to the inverse PCR product of *MAT1-1*. The *E. monoceras MAT1-2-1* gene encoded a putative protein of 345 amino acids. The protein also had high homology with the corresponding MAT1-2-1 of heterothallic *C. heterostrophus* (75% identity and 85% similarity), *A. alternata* (58% identity and 73% similarity), *L. maculans* (39% identity and 50% similarity), homothallic *C. kusanoi* (71% identity and 81% similarity), *P. tarda* (71% identity and 81% similarity), and *C. homomorphus* (75% identity and 83% similarity). The similarities were highest in the HMG box region. A putative 55-bp intron with fungal splice sites was identified in the HMG box at the same position as in the *MAT1-2-1* genes of *C. heterostrophus*, *P. teres*, *L. maculans*, *A. alternata*, and other ascomycetes (Fig. 2b).

Structural organization of the *Mat* locus in *E. monoceras*

All of the *MAT* idiomorphs and flanking common regions were analyzed using an inverse PCR method. Using DNA of strain 9.29 (*MAT1-2*), about 2.1 kbp of the upstream and 2.5 kbp of the downstream regions of the *MAT1-2-1* open reading frame (ORF) were obtained. The 5′ and 3′ terminal regions of the resultant sequence were expected to be common to those in *MAT1-1*. Primers designed based on both terminal sequences of the *MAT1-2* 5.6-kbp fragment successfully amplified the flanking regions of *MAT1-1-1* ORF in strain YM-1. The resultant 5.7-kb sequence of strain YM-1 (DDBJ/EMBL/GenBank Acc. No. AB622393) and 5.6-kb sequence of strain 9.29 (DDBJ/EMBL/GenBank Acc. No. AB622394) were compared to each other and appeared to be highly dissimilar in the idiomorphs (45.3% homology), whereas the flanking sequences were almost identical. Each idiomorph contained a single *MAT* gene, and the size of the idiomorph region was 1256 bp in *MAT1-1* and 1158 bp in *MAT1-2*. The length was comparable to that of closely related *C. heterostrophus* [*MAT1-1-1*: 1297 bp, and *MAT1-2-1*: 1171 bp (Turgeon et al. 1993); Fig. 3]. In *E. monoceras*, each idiomorph end is easy to determine, whereas in the related Dothideomycetes *P. nodorum* and *L. maculans* or Sordariomycetes *Fusarium oxysporum* and *Neurospora crassa*, the transition from idiomorph to common region is quite gradual. In such species, the idiomorphs are much longer [*F. oxysporum* (*MAT1-1*: 4618 bp and *MAT1-2*: 3850 bp), *P. nodorum* (*MAT1-1*: 4282 bp and *MAT1-2*: 4505 bp), *L. maculans* (*MAT1-1*: 3.5 kbp and *MAT1-2*: 4.0 kbp), or *N. crassa* (*MAT1-1*: 5.3 kbp and *MAT1-2*: 3.2 kbp)].

The 5′ flanking region of both *E. monoceras* idiomorphs contained an ORF with significant similarity to the *ORF1* genes of *C. heterostrophus*, *Cochliobolus ellisii*, and *L. maculans*. This ORF was similar to that of *Saccharomyces cerevisiae* YLR456 W (DDBJ/EMBL/GenBank Acc. No. U22383) and is conserved in the *Mat* loci of many heterothallic Dothideomycetes. In *E. monoceras*, 594-bp *ORF1* nucleotide sequences and deduced amino acid sequences were completely identical between opposite mating types (Fig. 4). In each strain, *ORF1* was 1424 bp distant from the idiomorph. In *C. heterostrophus* and *A. alternata*, idiomorphs and *ORF1* genes are separated by approximately 1.1 kbp and only 4 bp, respectively (Leubener-Metzger et al. 1997; Bennett et al. 2003). In *P. nodorum* or *L. maculans,* however, the 3′ region of the *ORF1* gene differed between opposite mating types, and the idiomorphs and *ORF1* sequences overlap (Figs. 3, 4). The dissimilarities resulted in the alteration of the ORF1 amino acid sequences in these species. These relatively longer idiomorphs tend to expand in the 5′ direction of *MAT* genes.

Transcription of genes in *Mat* locus

The expression patterns of *E. monoceras MAT* genes and flanking *ORF1* in various conditions were examined by RT-PCR. Total RNA was prepared from mycelia grown on MM and CM and from mycelia collected from infected plant tissue. RNA from mycelia grown in planta was obtained 4 days after inoculation when the lesion expansion and leaf etiolation were obvious. Transcribed RNAs from the *GPD* gene were used as controls. cDNA preparations were confirmed to be successfully carried out (Fig. 5, bottom). Intron-spanning primers 9 and 10 (for *MAT1-1*) and 11 and 12 (for *MAT1-2*) were designed and used to determine expression (Fig. 3). Transcripts of *MAT1-1-1* in strain YM-1 and of *MAT1-2-1* in strain 9.29 were detected under all growth conditions tested with approximately 50-bp size differences between amplicons from genomic DNA (Fig. 5). That is, *MAT* genes of these strains were expressed under culture conditions with high or low levels of nutrients and during pathogenic development. Sequencing of the cloned fragments confirmed the splicing events in both *MAT* genes. Transcripts of *E. monoceras ORF1* were also detected under all growth conditions tested (Fig. 5). No evident difference in expression was observed between the mating-type isolates.

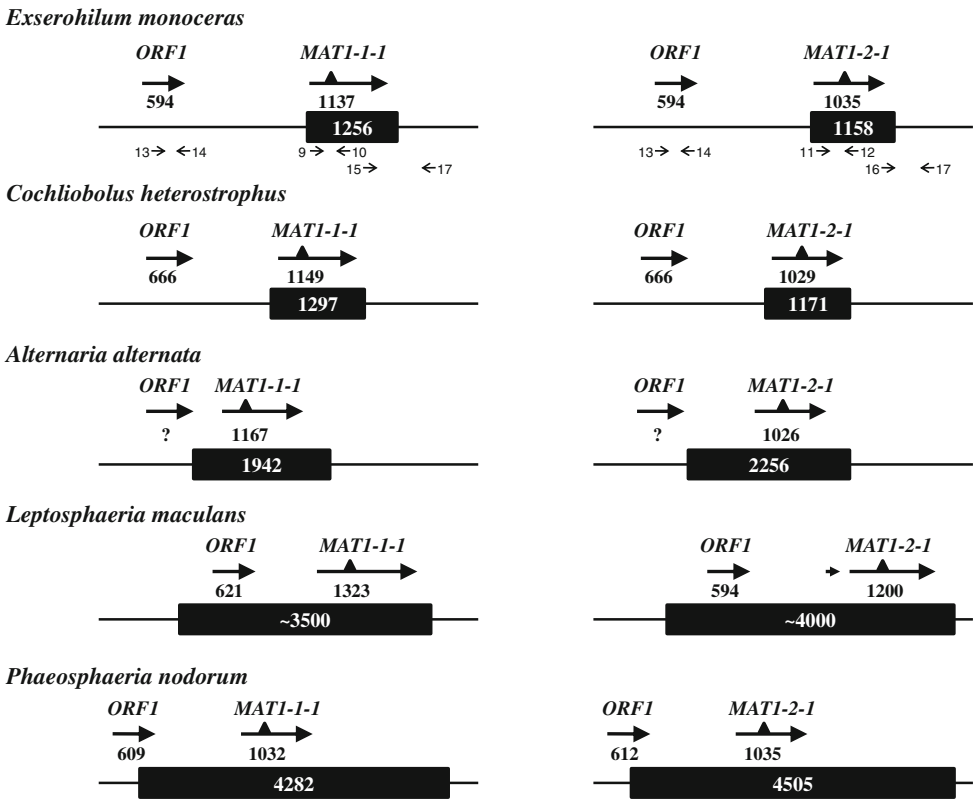

Fig. 3 Organization of *Mat* loci in *E. monoceras* and other heterothallic ascomycetes. *Black box* and *numbers within* Idiomorphs and their length, respectively, *arrows* genes of the *Mat* loci, *numbers under the arrows* sizes of the exons/open reading frames (ORFs), *triangles on arrows* introns. The complete sequence of the *Alternaria alternata ORF1* gene was unavailable. Primers 9–17 were noted in *E. monoceras*

Mating-type determination of field isolates by multiplex PCR assay

We developed a multiplex PCR assay to determine rapidly the mating type of *E. monoceras* Japanese field isolates. A *MAT1-1-1*-specific primer (MultiM1fr: primer 15 in Table 2), *MAT1-2-1*-specific primer (MultiM2fr: primer 16 in Table 2), and compatible flanking-specific primer (Multicommonrv: primer 17 in Table 2) were designed. The genomic DNAs from *E. monoceras* strains YM-1 and 9.29 were used as positive controls and *Exserohilum turcicum*, *E. rostratum*, and *C. heterostrophus* were used as negative controls for amplification with the three primers. A single 684-bp band from strain YM-1 and a 405-bp band from strain 9.29 were detected, as expected. No amplification was observed in the negative controls (data not shown). DNA from 35 Japanese isolates of *E. monoceras* (including strains YM-1 and 9.29) was analyzed. A single band of 684 or 405 bp was amplified in every isolate, and no isolate showed amplification of both bands (Fig. 6). Of the 35 isolates, 12 were *MAT1-1* and the remaining 23 isolates were *MAT1-2* (Table 1). Both mating types were found in the same subpopulation and from every host plant species isolated (Table 1). No obvious bias of the distribution of *MAT1-1* or *MAT1-2* isolates in the environment was observed (χ^2 value for 1:1 distribution of the mating types with Yates' correction for continuity was 2.86, $P > 0.05$).

Discussion

The genomic sequences of *MAT* genes and idiomorphs of *E. monoceras* were determined in this study. Degenerate primers designed based on the *MAT* sequences of other loculoascomycete-like fungi successfully detected *MAT* gene fragments in *E. monoceras*. The *Mat* locus of this fungus showed typical characteristics of heterothallic ascomycetes. Each idiomorph of *E. monoceras* contained the single protein MAT1-1-1 or MAT1-2-1 with α and HMG box domains, which have been found in all heterothallic fungal species to date. *ORF1* genes were located on the 5′ end of both *MAT* genes, as in other *Mat* loci of heterothallic loculoascomycetes.

The transcription test by RT-PCR revealed that genes in *Mat* locus were expressed during vegetative and pathogenic development in *E. monoceras*. Transcripts of *MAT* genes were detected from both mycelia grown in CM and MM. In *C. heterostrophus*, transcripts of *MAT* genes were detected only from cultures grown in MM, but not in CM (Leubener-Metzger et al. 1997). However, *L. maculans* expresses *MAT* genes independent of culture composition

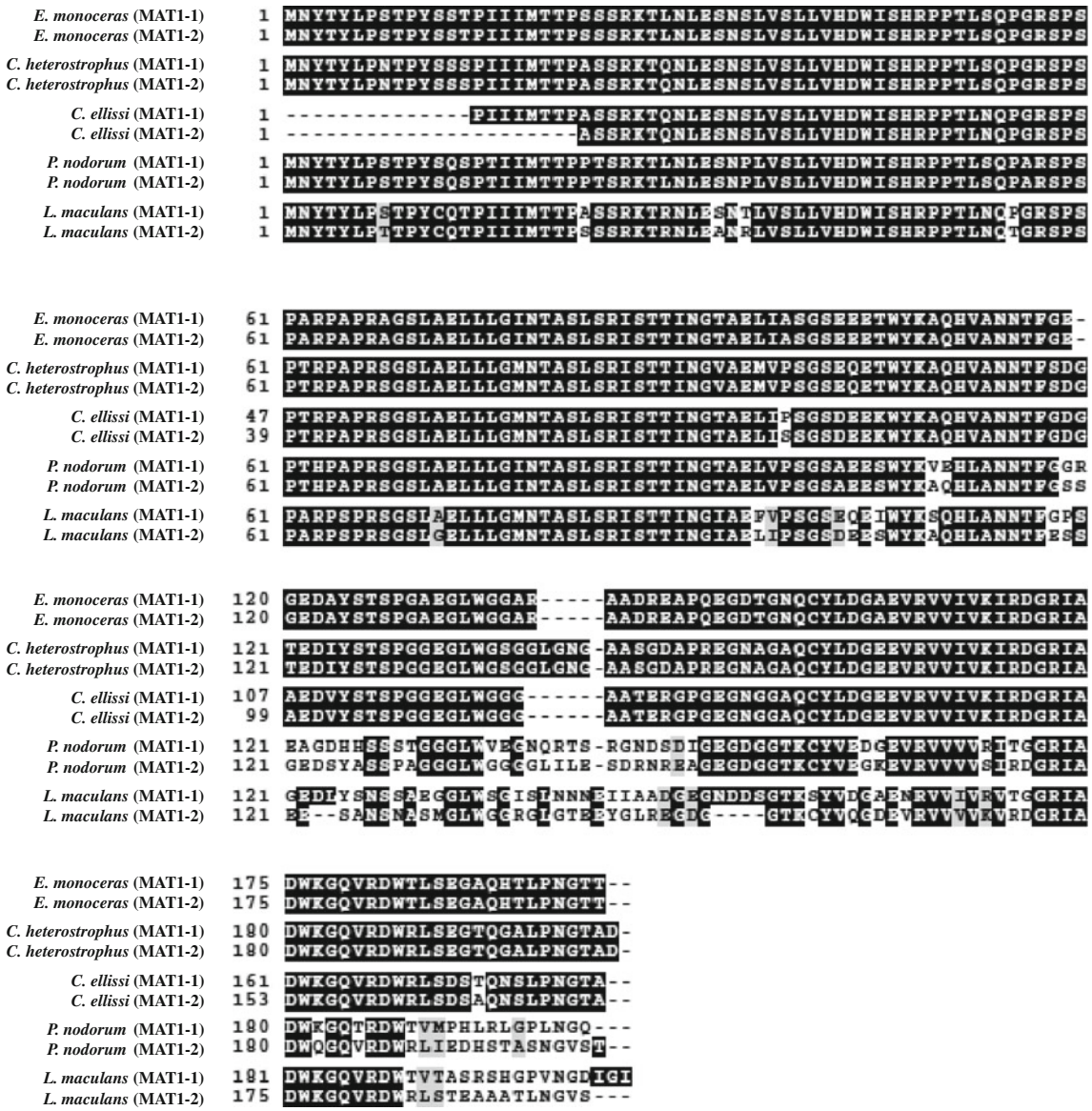

Fig. 4 Multiple alignment of deduced amino acid sequence of *ORF1* gene of both mating-type isolates of *E. monoceras* and other heterothallic ascomycetes. Complete sequences of *E. monoceras* (DDBJ/EMBL/GenBank Acc. Nos. AB622393 and AB622394), *Phaeosphaeria nodorum* (AAO31739 and AAO31741), and *Leptosphaeria maculans* (AAO37760 and AAO37756) and partial sequences of *Cochliobolus heterostrophus* (AAB82944 and AAB84003) and *Cochliobolus ellisii* (AAD33448 and AAD33450) are aligned

(Cozijnsen and Howlett 2003). The expression pattern of *E. monoceras MAT* genes was considered to fit the latter case. That is, the transcription of *E. monoceras MAT* genes was not tightly regulated depending on nutrient restriction conditions, unlike that of *C. heterostrophus*. Transcripts of *E. monoceras ORF1* were detected under all growth conditions used in this study. In the *C. heterostrophus MAT1-2* strain, deletion of *ORF1* did not affect pseudothecia and ascospore formation (Wirsel et al. 1998). The deletion of *S. cerevisiae* YLR456W, which is a homolog of fungal *ORF1*, resulted in no phenotypic change from wild type. Whether *ORF1* has role in *E. monoceras* is unknown, and it seems to be expressed constitutively. In *C. heterostrophus* and *L. maculans*, the expression of *ORF1* genes was also observed.

Primers designed for multiplex PCR tests successfully determined the mating type of Japanese field isolates of *E. monoceras* in a single reaction. The results suggest that both mating types were naturally distributed in the field and that the fungus had good sexual encounters, which implies that an absence of either mating type or the lack of *MAT* genes cannot explain why sexual states of *E. monoceras* have not been observed in the environment and have been only rarely reported in laboratories. In the multiplex PCR

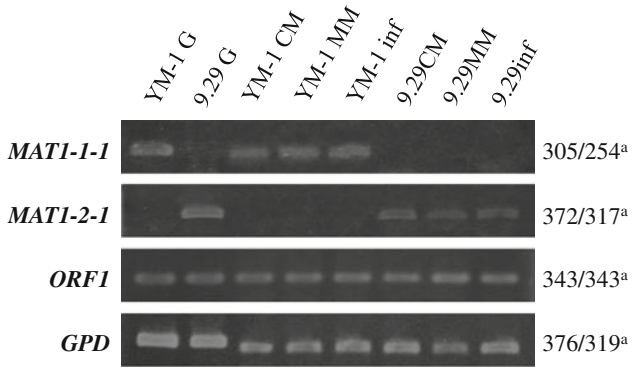

Fig. 5 Expression of *E. monoceras MAT* genes and the flanking *ORF1*. RNA transcripts from strains YM-1 (*MAT1-1*) and 9.29 (*MAT1-2*) were amplified by reverse transcriptase-PCR. *MAT1-1*-specific primer set 9 + 10, *MAT1-2*-specific primer set 11 + 12, and primer set 13 + 14 for *ORF1* were used (described in Table 2). As a control, an *E. monoceras GPD* (glucose-6-phosphate dehydrogenase) fragment was amplified. From the *left*, the following PCR templates were used: strain YM-1 genomic DNA, strain 9.29 genomic DNA, strain YM-1 RNA from liquid complete medium (CM), strain YM-1 RNA from liquid minimal medium (MM), strain YM-1 RNA from infected seedlings, strain 9.29 RNA from liquid CM, strain 9.29 RNA from liquid MM, and strain 9.29 RNA from infected seedlings. [a]*Numbers* indicate fragment sizes from genomic/cDNA templates, respectively

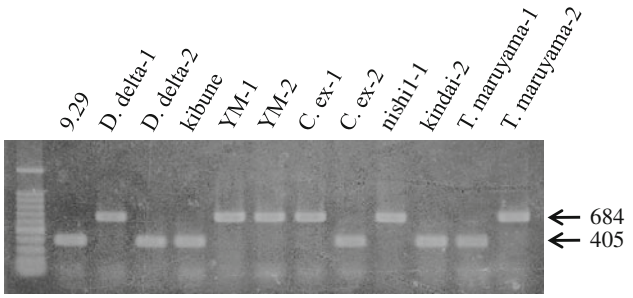

Fig. 6 Mating-type determination of representative *E. monoceras* field isolates by multiplex PCR assay. PCR was performed with primer set 15 + 16 + 17 as described in Table 2 and Fig. 3. The amplicons of 684 or 405 bp indicate the *MAT1-1* or *MAT1-2* genotype, respectively. *Slanted labels* indicate strain names: their details are given in Table 1

mating-type test, eight isolates of *MAT1-1* were chosen and crossed to *MAT1-2* isolate 9.29. Four isolates formed mature ascocarps and ascospores (Morita et al. 2011). Combined with the result reported by Morita et al. (2011), the knowledge obtained in this study will provide potent approaches for conventional genetic manipulation of *E. monoceras*.

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