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Magnaporthe oryzae MTP1 gene encodes a type III transmembrane protein involved in conidiation and conidial germination*

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Abstract: In this study the *MTP1* gene, encoding a type III integral transmembrane protein, was isolated from the rice blast fungus *Magnaporthe oryzae*. The Mtp1 protein is 520 amino acids long and is comparable to the Ytp1 protein of *Saccharomyces cerevisiae* with 46% sequence similarity. Prediction programs and *MTP1-GFP* (green fluorescent protein) fusion expression results indicate that Mtp1 is a protein located at several membranes in the cytoplasm. The functions of the *MTP1* gene in the growth and development of the fungus were studied using an *MTP1* gene knockout mutant. The *MTP1* gene was primarily expressed at the hyphal and conidial stages and is necessary for conidiation and conidial germination, but is not required for pathogenicity. The Δ*mtp1* mutant grew more efficiently than the wild type strain on non-fermentable carbon sources, implying that the *MTP1* gene has a unique role in respiratory growth and carbon source use.

Key words: *Magnaporthe oryzae*, *Magnaporthe grisea*, *MTP1*, cDNA, Gene knockout, Conidial germination
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INTRODUCTION

Magnaporthe oryzae, distinguished from *Magnaporthe grisea* (Couch and Kohn, 2002), is a major fungal pathogen of rice and other cereals, causing devastating rice blast disease throughout the world (Ou, 1985). In China more than 3.8×10^6 ha of rice has been exposed to this disease every year since 1990. Much has been learned of the molecular mechanisms regulating pre- and post-penetration events in the development and pathogenicity of *M. oryzae*, such as those involved in appressorium formation and invasion growth (Lee and Dean, 1993; Xu and Hamer, 1996; Choi and Dean, 1997; DeZwaan *et al*., 1999; Clergeot *et al*., 2001; Kim *et al*., 2005; Zhao *et al*., 2005; Veneault-Fourrey *et al*., 2006a; Odenbach *et al*., 2007). Among those known important genes, Pth11 is a plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues (DeZwaan *et al*., 1999). Pls1 is another transmembrane protein localized in plasma membranes and vacuoles of appressoria, required for the penetration of the rice leaf by the fungal pathogen (Clergeot *et al*., 2001; Veneault-Fourrey *et al*., 2006b). However, the functions of most transmembrane proteins in this fungal pathogen have not been fully characterized. The plasma membrane is an interface interacting with plant cells when the pathogen penetrates into or grows in the plant. The signals outside of the cell are imported through the cell membrane to activate fungal developmental events, which may result in cell differentiation or growth (DeZwaan *et al*., 1999; Gronover *et al*., 2001; Dean *et al*., 2005). The vacuole membrane is involved in autophagy, which is required for the pathogenicity of the rice blast fungus (Veneault-Fourrey *et al*., 2006a; Liu *et al*., 2007). The proteins integrated into the mito-

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chondrial membrane are also necessary for the function of mitochondrion. The mitochondrion is an important organelle and is the site at which fatty acid β-oxidation occurs, which is necessary to synthesize glycerol in the appressorium from storage products (Dean *et al*., 2005). Glycerol is necessary in order to generate turgor pressure in an appressorium, which is required for plant penetration and pathogenicity (de Jong *et al*., 1997).

MTP1 gene, encoding a type III integral transmembrane protein, is one of hundreds of genes isolated from the *M. oryzae* appressorium cDNA library in our previous study (Lu *et al*., 2005a). Little is known about the functional and pathological significance of the Mtp1 protein in the rice blast fungus, or its homologues in other filamentous fungi. In this study we report the molecular structure of the *MTP1* gene which encodes a transmembrane protein with 8 membrane helices, its expression profile during growth and development and its function which was elucidated through gene knockout experiments in *M. oryzae*.

MATERIALS AND METHODS

Fungal strain, DNA/RNA isolation and manipulation

The *M. oryzae* wild type strain, Guy-11, and mutants (transformants and an *MTP1*-knockout mutant generated from Guy-11) were cultured on complete medium (CM) plates (Talbot *et al*., 1993) at 25 °C with a 14-h light and 10-h dark cycle using fluorescent lights. Studies involving a cross with strain 2539 were conducted on oat meal agar (OMA) medium (30 g oatmeal in 1 L distilled water). DNA and total RNA extraction, polymerase chain reaction (PCR), restriction digest reaction, gel electrophoresis and ligation reaction were carried out following standard procedures (Sambrook *et al*., 2002).

Isolation and sequence analysis of *MTP1*

The cDNA fragment containing the full coding sequence of the *MTP1* gene was cloned from the cDNA library (Lu *et al*., 2005b) by PCR using the primers s148RNAp1 (5′-CCACCACGGCTTCAATCCCGCGAC-3′) and s148RNAp2 (5′-GATGCACATCGGTGCAGAGCTGTC-3′), cloned into the T-vector, pUCm-T (Sangon, Shanghai, China), and sequenced on an ABI377 DNA sequencer (Invitrogen, USA). The DNA fragment containing the *MTP1* gene was amplified from Guy-11 genomic DNA using the primers s148p1 (5′-GTTTGGTTAATTGTCTTCCCGGCTGTTTTG-3′) and s148p2 (5′-CACCCCCTTCCCGAGTCTTGATTTGTTGT-3′), cloned into the pCR-XL®-TOPO® vector (Clontech, USA), and sequenced.

The putative protein Mtp1 was predicted for homologies by using the Blastp (Altschul *et al*., 1997) and ClustalW (http://www.ebi.ac.uk/clustalw/) (Thompson *et al*., 1994), for subcellular localization by TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP-1.1/) (Emanuelsson *et al*., 2000) and WoLFPSORT (http://wolfpsort.seq.cbrc.jp) (Horton *et al*., 2006), for the presence and location of signal peptide cleavage sites by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Dyrløv Bendtsen *et al*., 2004), and for protein structure by Predictprotein (http://www.predictprotein.org/) (Rost *et al*., 2004).

Construction of the *MTP1* knockout vector

The knockout vector, pBS-MTP1, was constructed by inserting two flanking sequences of *MTP1* gene into the pBS-HPH2 vector. pBS-HPH2, an intermediate vector, was constructed by inserting a 1344-bp *Sal*I-*Bam*HI PCR fragment containing a hygromycin phosphotransferase (*HPH*) gene expression cassette into the *Sal*I-*Bam*HI sites of the pBS(+) vector (Stratagene, USA). The *HPH* gene expression cassette fragment was prepared by PCR amplification from the plasmid pCB1003 (Carroll *et al*., 1994) by using the primers 5′-CCggatccTGGAGGTCAACACATCAAT-3′ and 5′-CCgtcgacCTACTCTATTCCTTTGCCCTCG-3′. A 1240-bp *Kpn*I-*Sal*I downstream flanking sequence fragment of *MTP1*, amplified from genomic DNA using the primers s148dnp1 (5′-AAggtaccTGCTGCTGTTACCGCGAAGGATT-3′) and s148dnp2 (5′-AAgtcgacAAGAGGCGGCGAAGAGGAAGTTAC-3′), was firstly inserted into the *Kpn*I-*Sal*I sites of pBS-HPH2 and then another 1231-bp *Spe*I-*Not*I upstream flanking sequence fragment obtained by PCR with the primers s148upp1 (5′-AAggtaccTGCTGCTGTTACCGCGAAGGATT-3′) and s148upp2 (5′-AAgtcgacAAGAGGCGGCGAAGAGGAAGTTAC-3′) was inserted into the *Spe*I-*Not*I sites of pBS-HPH2 to generate the knock-

out vector pBS-MTP1. The 3.5-kb *Hin*dIII digested pBS-MTP1 vector was used to transform protoplasts of Guy-11.

Fungal transformation and identification of *MTP1* knockout mutants

The transformation procedure, including protoplast generation, was conducted as described previously (Lu *et al*., 2007a). Transformants were recovered and selected in CM with 20% (w/v) sucrose and 200 μg/ml hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany). Transformants were further confirmed by passing through a second selection in the hygromycin B media.

Initial identification of the knockout mutants was performed by PCR using primers internal to *MTP1* (s148ckp1: 5′-CGGCGTCGTCCCTCGTGTTGG-3′ and s148ckp2: 5′-TCGCGGCGCTGGGATTCTGC-3′), resulting in amplification of an 832-bp fragment of the *MTP1* gene. Putative knockout transformants identified by PCR screens were purified by single conidium isolation, and confirmed further by Southern hybridization analysis. Genomic DNAs from transformants were prepared, and cut with the *Eco*RV restriction enzyme for Southern blot analysis. A 547-bp DNA fragment located in the upstream flanking sequence of *MTP1* gene was obtained by PCR from genomic DNA with the primers s148sb2p1 (5′-CGAAACGGCGAAGACGAA-3′) and s148sb2p2 (5′-GCCGCCGCCCGCCAACAT-3′), and used as a probe for Southern blot. Southern blot hybridizations were conducted using the DIG high prime DNA labeling and detection starter kit I (Roche Diagnostics GmbH, Mannheim, Germany).

Construction and expression of the *MTP1-GFP* fusion protein

A 1.4-kb *HPH* gene fragment was amplified from the plasmid pCB1003 with the primers p1 (5′-ACgcggccgcGGAGGTCAACACATCAATG-3′) and p2 (5′-CCaggcctGGTCGGCATCTACTCTATTC-3′) and cloned into the *Not*I-*Stu*I sites of pEGFP (Clontech, USA), resulting in generation of the plasmid pEGFP-HPH. A 1534-bp upstream sequence of *MTP1* was amplified from Guy-11 genomic DNA with the PCR primers s148pro-p1 (5′-CTGAggatccAGCAGACGACGCGGAAGAAGAGATGAGAC-3′) and s148pro-p2 (5′-GTGTggatccCGCGGTTTAGATGCTGGTAGTATTTTG-3′) and cloned into the *Bam*HI site of pEGFP-HPH to generate pEGFP-MTP1. In pEGFP-MTP1, the green fluorescent protein (*GFP*) construct was under the control of the native *MTP1* promoter.

The promoter sequence of the *NAR* gene (Lu *et al*., 2007b) was amplified from Guy-11 genomic DNA using the PCR primers 5′-CaggatccGGGAAGCGATTGCGTT-3′ and 5′-GGTGccatggTGTCGGTTGTGGTG-3′, and inserted into the *BamH*I-*Nco*I sites of pEGFP to produce the pEGFP-NAR vector. The *GFP* construct without a stop codon (TAA) under the control of the *NAR* promoter was amplified from pEGFP-NAR with the primers 5′-CTgtcgacGGGAAGCGATTGCGTTT-3′ and 5′-TTccgggCTTGTACAGCTCGTCCATGC-3′, and cloned into the *Sal*I-*Sma*I sites of the pBS(+) vector to generate the vector pBS-NE. Subsequently the coding domain of the *MTP1* fragment without the initiation codon (ATG) was amplified using the primers 5′-TTcccgggGCGTGGTCGAGAATTGGGAT-3′ and 5′-TTctagaTTTCACGCAACCCGATCGCTAT-3′ from *MTP1* cDNA and inserted into the *Sma*I-*Xba*I sites of pBS-NE to generate pBS-NEM. Then a 0.9-kb *BAR* gene fragment was obtained from pBARKS1 (Pall and Brunelli, 1993) via PCR using the primers 5′-AatctagAGAAGATGATATTGAAGGA-3′ and 5′-AatctagaCTAAATCTCGGTGACGGGC-3′, and cloned into the *Xba*I site of pBS-NEM to generate pEGFP-MTP2. In the pEGFP-MTP2 plasmid, the resulting *MTP1-GFP* fusion construct was under the control of the *NAR* promoter.

The resulting vector, pEGFP-MTP1, was transformed into protoplasts of Guy-11 and selected with 200 μg/ml hygromycin B. Another resulting vector, pEGFP-MTP2, linearized with *Kpn*I, was transformed into protoplasts of the Δ*mtp1* mutant and selected with 150 μg/ml glufosinate-ammonium (Yongnong Ltd., Zhejiang, China). Hygromycin-resistant transformants or glufosinate-ammonium-resistant transformants were individually confirmed by DNA gel blot analysis. The expression of *GFP* was examined using an Olympus-BX51 fluorescence microscope with a cooled CCD camera DP50 (Olympus, Japan) and Zeiss LSM-510 laser scanning microscope (Carl Zeiss, Germany).

Growth characteristics of the Δ*mtp1* mutant

Vegetative growth of both the *M. oryzae* Δ*mtp1* mutant and Guy-11 strains was examined following a previously described procedure (Lu *et al*., 2007a) in CM medium, CM-N medium (CM medium without the nitrogen source), CM-C medium (CM medium without the carbon source), CM-hyperosmotic medium (CM medium with 1 mol/L NaCl) (Talbot *et al*., 1993) and YPEG medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (v/v) ethanol, 3% (v/v) glycerol, 2% (w/v) agar). With the same procedure, the influences of Cu^{2+} ($CuSO_4$, 1.0×10^{-4} mol/L) and Fe^{3+} ($FeCl_3$, 1.0×10^{-4} mol/L) in 1/4 YG medium (0.13% (w/v) yeast extract, 0.5% (w/v) glucose, 1.5% (w/v) agar) on vegetative growth of the mutant were evaluated. Conidiation, conidial germination and appressorium development of the mutant and Guy-11 were also counted according to a previously described procedure (Lu *et al*., 2007a). Appressorium turgor of the mutant was estimated using incipient cytorrhysis (cell collapse) assay (Howard *et al*., 1991).

Pathogenicity assays

The inoculation method was carried out as previously reported (Lu *et al*., 2007a). Two-week-old seedlings of rice (*Oryza sativa* cv. CO-39) or 8-day-old barley (*Hordeum vulgare* cv. ZJ-8) were spray inoculated with conidial suspension (1×10^5 conidia/ml) and then placed in a controlled environment chamber with a 14-h light and 10-h dark cycle using fluorescent lights for 7 d (rice) or 4 d (barley). Disease severity was recorded according to the method proposed by Bonman *et al*.(1986).

RESULTS

Isolation of *MTP1*

An EST (expressed sequence tag) (GenBank_Accn: CK828223), which encodes an *M. oryzae* transmembrane protein 1 (Mtp1) and is expressed in hyphae, conidium and appressorium, was identified from ESTs of a suppression subtractive hybridization cDNA library of the *M. oryzae* strain Guy-11 (Lu *et al*., 2005a). After searching the *M. oryzae* database (http://www.broad.mit.edu/), this EST was found to correspond to a hypothetical protein, MG05336.5, in strain 70-15. Then the cDNA fragment containing the full coding sequence was cloned from the Guy-11 appressorium cDNA library (Lu *et al*., 2005b) using high fidelity PCR and subsequently sequenced (GenBank_Accn: DQ085779). The cDNA obtained was 1982-bp long and included 1560 bp of the coding sequence, 195 bp of the 5′ untranslated region and 227 bp of the 3′ untranslated region. The 6073-bp *MTP1* genomic DNA fragment was also cloned from genomic DNA of Guy-11 by long distance PCR and sequenced (GenBank_Accn: EF525179). The nucleotide sequence of the genomic *MTP1* gene contains a putative 1853-bp open reading frame with three introns that are 140-bp, 89-bp and 61-bp long. The coding sequence of *MTP1* encodes a predicted mature polypeptide of 520 amino acids. This polypeptide sequence shows 46% identity in sequence to the Ytp1 protein of *Saccharomyces cerevisiae* (West *et al*., 1996), a type III transmembrane protein, as predicted by Blastp and ClustalW (Thompson *et al*., 1994; Altschul *et al*., 1997) during sequence similarity searches. The Mtp1 protein also shows high similarity to many proteins in filamentous fungi, such as predicted protein (GenBank_Accn: CAP73000) in *Podospora anserine* (63% in identity), predicted protein (GenBank_Accn: EAA28954) in *Neurospora crassa* (62%) and predicted protein (GenBank_Accn: XP_001225052) in *Chaetomium globosum* (62%). However, these homologues in filamentous fungi have not been studied until now.

Predictprotein (PHDhtm) (Rost *et al*., 1996) predicted that the Mtp1 protein is likely to be a type III (polytopic) transmembrane protein with 8 membrane helices. WoLFPSORT (Horton *et al*., 2006) predicted that the Mtp1 protein is a transmembrane protein, probably located at the plasma membrane. However, TargetP 1.1 (Emanuelsson *et al*., 2000) predicted that Mtp1 protein is most likely to be located in the mitochondrion. SignalP 3.0 (Dyrløv Bendtsen *et al*., 2004) predicted that the Mtp1 protein is not a secretory protein (by neural network). These predicted results suggested that the Mtp1 protein is a transmembrane protein, possibly located at the mitochondrial membrane or the plasma membrane.

Analysis of *GFP* expression under the control of the native *MTP1* promoter

A *GFP* expression vector (pEGFP-MTP1), under the control of the native *MTP1* promoter, was

constructed and transformed into the wild type *M. oryzae* strain. Then the differential expression pattern of the *MTP1* gene at various developmental stages was analyzed using seven transformants. Observation of GFP fluorescence in transformants under an Olympus-BX51 epifluorescence microscope showed that GFP was expressed in hyphae and spores. However, it was faintly expressed in germinating spores and appressoria of *M. oryzae* (Fig.1, see Page 516). This implied that the *MTP1* gene is more highly expressed and active in the hyphal and conidial stages than in the appressorial stage.

Knockout of *MTP1*

A gene knockout strategy was employed to determine the function of *MTP1*. A knockout vector, pBS-MTP1 (Fig.2a), containing the *HPH* gene cassette flanked by 5′ and 3′ ends of the *MTP1* gene was constructed. One *MTP1* knockout mutant (F7) out of nine hygromycin resistant transformants was determined initially by PCR using primers internal to *MTP1*, and then by DNA gel blot analysis, to have undergone gene deletion. In DNA gel blot analysis a 9.0-kb fragment was detected in the transformant F7 in contrast with a 4.9-kb fragment found in the wild type strain (Fig.2b). The band shift from 4.9-kb to 9-kb showed that the *MTP1* gene had been deleted in the transformant F7 (namely Δ*mtp1* mutant) and that homologous recombination had occurred at a single site.

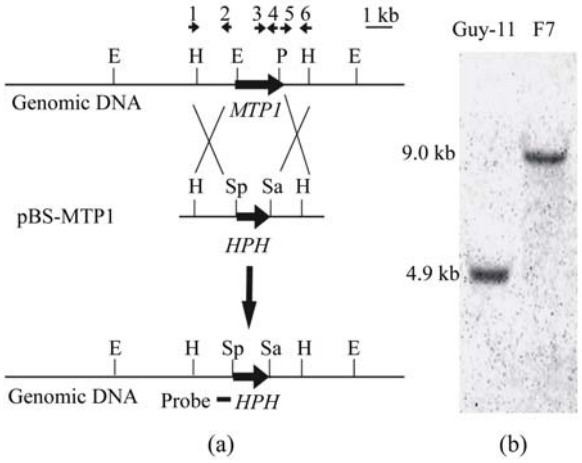

Fig.2 Deletion of the *MTP1* gene in *M. oryzae*. (a) *MTP1* locus and knockout vector (pBS-MTP1). Large arrows indicate orientations of the *MTP1* and *HPH* genes. The positions and orientations of primers s148upp1, s148upp2, s148ckp1, s148ckp2, s148dnp1 and s148dnp2 are labeled with small arrows 1, 2, 3, 4, 5 and 6. The *Hin*dIII digested fragment containing the gene deletion construct was excised, purified by gel electrophoresis and used to transform *M. oryzae* Guy-11 protoplasts. E=*Eco*RV, H=*Hin*dIII, P=*Pvu*I, Sa=*Sal*I, Sp=*Spe*I; (b) DNA gel blot analysis of Δ*mtp1* mutant. All genomic DNA samples were digested with *Eco*RV, fractionated and probed with a 547-bp fragment located in the upstream flanking sequence of *MTP1* shown in Fig.2a. As expected, a 9-kb band was detected in Δ*mtp1* mutant F7 in contrast with a 4.9-kb band in the wild type strain Guy-11

Analyses of *MTP1-GFP* fusion expression

In order to analyze Mtp1-GFP localization in a cell, a *MTP1-GFP* fusion expression vector (pEGFP-MTP2), under the control of the *NAR* promoter, was constructed and transformed into the Δ*mtp1* mutant. When observed under a normal fluorescence microscope, the GFP fluorescence was observed strongly in the hyphal cytoplasm and weakly in the vacuoles of transformants (Fig.3a). However, under a confocal laser scanning microscope the GFP fluorescence was observed also in the hyphal vacuoles (Fig.3b) and in conidial cytoplasm with unidentified minute dot-like structures (Fig.3c). These observations of GFP fluorescence in transformants showed that GFP was located in the cytoplasm associated with the membrane of vacuoles or other membranes in the cytoplasm (Fig.3). Considering the above-mentioned program-predicted results, the *MTP1-GFP* fusion expression results implied that the possible locations of the Mtp1 protein are membranes on several organella in the cytoplasm.

Characterization of the Δ*mtp1* mutant

The growth in colony diameters of the Δ*mtp1* mutant and the wild type strain was comparable in the CM medium, the CM-N medium, the CM-C medium and the CM-hyperosmotic medium ($P \leq 0.05$). The colony growth of the Δ*mtp1* mutant on 1/4 YG medium was not slowed by adding Cu^{2+} ($CuSO_4$, 1.0×10^{-4} mol/L) and Fe^{3+} ($FeCl_3$, 1.0×10^{-4} mol/L) ($P \leq 0.05$), in contrast to that of the wild type strain. The colony color of the mutant and wild type strains became darker on 1/4 YG medium following the addition of $CuSO_4$. Interestingly the Δ*mtp1* mutant [(4.44±0.09) cm in colony diameter at 12 days post-inoculation (dpi)] grew faster than the wild type strain [(3.55±0.05) cm at 12 dpi] or *MTP1* rescued strain MTG-3, a Δ*mtp1* mutant in which the

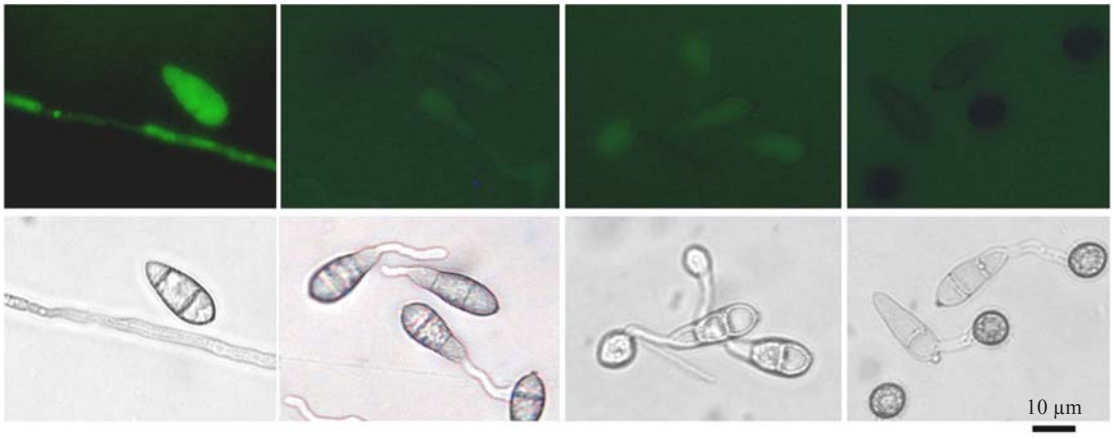

Fig.1 Differential expression pattern of *MTP1*. The green fluorescence emitted by GFP protein, which was expressed under the control of the *MTP1* promoter, was bright in hyphae and conidia (a), faint in germinating conidia at 2 hours post-inoculation (hpi) (b) and forming appressoria at 4 hpi (c) and invisible in mature appressoria at 24 hpi (d) of *M. oryzae*. The mycelia, conidia and appressoria were observed by fluorescence microscopy (top) and by light microscopy (bottom) in each panel

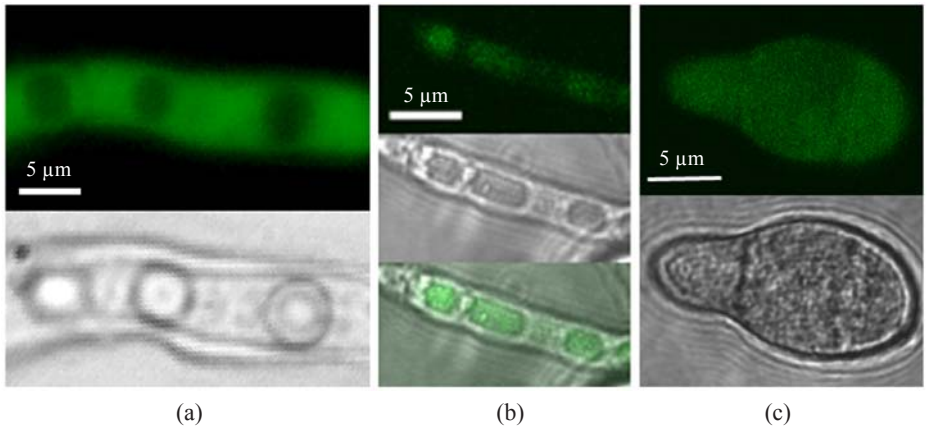

Fig.3 Cellular localization of Mtp1. (a) The mycelia were observed under Olympus-BX51 fluorescence microscopy (top, eGFP; bottom, light); (b) The mycelia were observed under a laser scanning microscope (top, eGFP; middle, light; bottom, merge); (c) The conidia were observed under a laser scanning microscope (top, eGFP; bottom, light). *GFP-MTP1* fusion protein appeared in the cytoplasm of the *M. oryzae* cell, associated with the membrane of vacuoles or other organella in the cytoplasm

Fig.4 Lesions caused by *M. oryzae* on the leaves of rice seedlings. These leaves were photographed 7 d after the rice seedlings (cv. CO-39) were individually spray-inoculated with conidia (1×10^5 conidia/ml) of the wild type strain Guy-11, Δ*mtp1* mutant or sprayed with 0.2% (w/v) gelatin (control)

MTP1 gene was re-introduced via an *MTP1-GFP* expression vector (pEGFP-MTP2) [(3.63±0.07) cm at 12 dpi] on the YPEG medium (complete medium containing ethanol and glycerol as a sole non-fermentable carbon source) ($P\leq0.05$). Also, the colony color of the mutant was darker than that of the wild type strain in the YPEG medium. Therefore the Δ*mtp1* mutant was devoid of colony growth defects in these solid media, and the *MTP1* gene is not essential for the mitochondrial respiratory function. In mating experiments, the Δ*mtp1* mutant formed normal perithecia with viable ascospores after 4 weeks of

incubation with the opposite mating-type strain 2539. However, conidiogenesis in the Δ*mtp1* mutant was reduced significantly. The mutant produced $(0.70\pm0.05)\times10^4$ conidia/mm^2 on the CM medium plate, approximately close to half of that produced by the wild type strain [$(1.30\pm0.08)\times10^4$ conidia/mm^2] or *MTP1* rescued strain MTG-3 [$(1.20\pm0.07)\times10^4$ conidia/mm^2] ($P\leq0.05$). In the germination assay, conidia of the Δ*mtp1* mutant were slower to germinate than those of the wild type strain (Table 1). Two hours after being placed onto plastic covers, only 21.1% of the conidia of the Δ*mtp1* mutant germinated compared to 90.2% of those of wild type strain. Four hours later the rate of germ tube emergence from the Δ*mtp1* mutant was still lower than that of the wild type strain. The rate of later appressorium formation in the Δ*mtp1* mutant was also delayed (Table 1). Six hours post-inoculation (hpi), the rate of appressorium formation in the mutant was 66.3%, while in the wild type strain it was 86.6%. However, 24 h later the rate of appressorium formation was similar in both. In *MTP1* rescued strain MTG-3, the defects of the Δ*mtp1* mutant in conidium germination and appressorium formation were recovered (Table 1). To evaluate the role of *MTP1* in appressorium turgor, we measured the turgor of the mature appressorium using the incipient cytorrhysis test described by Howard *et al.*(1991). The rate of collapsed appressoria in a series of glycerol solutions of varying strengths did not differ significantly between the Δ*mtp1* mutant and the wild type strain ($P\leq0.05$).

Table 1 Analysis of conidial germination and appressorium formation

Strain	Conidial germination (%)[1]		Appressorium formation (%)[2]	
	2 hpi	4 hpi	6 hpi	24 hpi
Guy-11	90.2±1.9 a	98.5±0.2 a	86.6±5.2 a	99.3±0.4 a
F7	21.1±0.6 b	91.0±1.0 b	66.3±4.0 b	96.2±1.1 a
MTG-3	89.3±2.2 a	96.5±1.8 a	87.5±5.8 a	97.6±0.8 a

The conidia suspension of the wild type strain Guy-11, the Δ*mtp1* mutant F7 and the *MTP1*-rescued strain MTG-3 (1×10^5 conidia/ml, 20 μl) was droplet inoculated on plastic coverslips at 25 °C. [1]The percentage of conidia that had elaborated a germ tube was counted at 2 hpi or 4 hpi; [2]The percentage of conidia forming appressoria was counted at 6 hpi or 24 hpi. The experiments were repeated three times with more than 300 conidia counted each time. The same lowercase letters in each column denote that the difference estimated by the Duncan's test ($P\leq0.05$) is not significant

Effect of *MTP1* gene deletion on pathogenicity

Infection assays were performed to assess the ability of the Δ*mtp1* mutant to cause disease in barley and susceptible rice. In conidial sprays no significant difference in disease symptoms could be observed on plants inoculated with either the Δ*mtp1* mutant or the wild type strain (Fig.4, see Page 516).

DISCUSSION

In this study we have cloned and sequenced the *MTP1* gene, a *YTP1* homologue in *M. oryzae*. The Mtp1 protein shares 46% amino acid identity with a previously identified *S. cerevisiae* Ytp1 protein (West *et al*., 1996). The *YTP1* gene encodes a type III transmembrane protein, which is not essential for the growth and development of yeast (West *et al*., 1996). The computer programs predicted that the Mtp1 protein is a type III transmembrane protein possessing 8 membrane helices. *MTP1-GFP* fusion expression experiments showed that the Mtp1 protein might be located on several membranes in the cytoplasm.

The *MTP1* gene is not essential for the colony growth of *M. oryzae*. The Δ*mtp1* mutant does not confer sensitivity to starvation, Cu^{2+} or Fe^{3+}, and exhibits no detectable osmoregulation defect. Moreover, the Δ*mtp1* mutant does not lose its mating ability to cross with the opposite mating-type strain. However, in contrast to those in Δ*ytp1* mutant of yeast, which lacked a detectable growth phenotype (West *et al*., 1996), the phenotypic effects displayed through deletion of the *MTP1* gene in *M. oryzae* were marked by a reduction in conidiation and a delay in conidium germination and appressorium formation. The delay in appressorium formation may be due to a delay in conidium germination. The conidial formation and conidium germination in the rice blast fungus are a complicated process, affected by many known genes, such as a blue light receptor gene *SMO1* (Hamer and Givan, 1990), adenylate cyclase gene *MAC1* (Adachi and Hamer, 1998), two PAK kinase genes *CHM1* and *MST20* (Li *et al*., 2004), *MgWC-1* (Lee *et al*., 2006), and autophagy related genes *MgATG1* and *MgATG8* (Veneault-Fourrey *et al*., 2006a; Liu *et al*., 2007). In *Aspergillus oryzae* or *M. oryzae*, conidium formation and conidial germination rely on endogenous sources for nutrient supply through autophagy (Kikuma *et al*.,

2006; Liu *et al*., 2007). The mitochondrion is an organelle that functions at the turnover of endogenous nutrient sources and supplies adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle, β-oxidation and oxidative phosphorylation. Recently it was found that Mtp1 might interact with Pls1 in the appressorium of *M. oryzae* (personal communication, Marc-Henri Lebrun). Pls1 is a transmembrane protein (tetraspanins), only localized in plasma membranes and vacuoles of appressoria and required for the formation of the penetration peg at the base of the appressorium, probably through re-establishing cell polarity (Clergeot *et al*., 2001; Veneault-Fourrey *et al*., 2006b). And Mtp1 protein possibly plays its role in the function of mitochondrion or vacuole in the turnover of nutrient sources or the supply of energy sources, which could be used during the conidial formation stage and the early stage of conidial germination, or play its role through interacting with Pls1 protein at the stage of appressorium.

As the Δ*mtp1* mutant could use ethanol and glycerol as its sole carbon sources and produce similar appressorium turgor as the wild type strain, the Δ*mtp1* mutant exhibits no defects in the mitochondrial respiratory function and fatty acid β-oxidation. Interestingly the Δ*mtp1* mutant seems to use ethanol and glycerol more readily than the wild type strain, as it grows faster than the wild type strain in non-fermentable carbon sources. In yeast Δ*tpk2* mutants also grew better than the wild type strain in non-fermentable carbon sources, and the protein kinase A (PKA) catalytic subunit Tpk2 protein, which represses transcription of genes involved in high-affinity iron uptake, showed a unique role in respiratory growth and carbon source use (Robertson *et al*., 2000). The Mtp1 protein may have a similar function to the Tpk2 protein; however, more direct experimental analyses are required to confirm this.

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