**MoPex19, which Is Essential for Maintenance of Peroxisomal Structure and Woronin Bodies, Is Required for Metabolism and Development in the Rice Blast Fungus**

Ling Li¹,²*, Jiaoyu Wang²*, Zhen Zhang², Yanli Wang², Maoxin Liu¹,², Hua Jiang³, Rongyao Chai², Xueqin Mao², Haiping Qiu², Fengquan Liu¹*, Guochang Sun²*

¹Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Nanjing, China, ²State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Institute of Plant Protection Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

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**Abstract**

Peroxisomes are present ubiquitously and make important contributions to cellular metabolism in eukaryotes. They play crucial roles in pathogenicity of plant fungal pathogens. The peroxisomal matrix proteins and peroxisomal membrane proteins (PMPs) are synthesized in the cytosol and imported post-translationally. Although the peroxisomal import machineries are generally conserved, some species-specific features were found in different types of organisms. In phytopathogenic fungi, the pathways of the matrix proteins have been elucidated, while the import machinery of PMPs remains obscure. Here, we report that MoPEX19, an ortholog of ScPEX19, was required for PMPs import and peroxisomal maintenance, and played crucial roles in metabolism and pathogenicity of the rice blast fungus *Magnaporthe oryzae*. MoPEX19 was expressed in a low level and Mopex19p was distributed in the cytoplasm and newly formed peroxisomes. MoPEX19 deletion led to mislocalization of peroxisomal membrane proteins (PMPs), as well peroxisomal matrix proteins. Peroxisomal structures were totally absent in Δmopex19 mutants and woronin bodies also vanished. Δmopex19 exhibited metabolic deficiency typical in peroxisomal disorders and also abnormality in glyoxylate cycle which was undetected in the known mopex mutants. The Δmopex19 mutants performed multiple disorders in fungal development and pathogenicity-related morphogenesis, and lost completely the pathogenicity on its hosts. These data demonstrate that MoPEX19 plays crucial roles in maintenance of peroxisomal and peroxisome-derived structures and makes more contributions to fungal development and pathogenicity than the known MoPEX genes in the rice blast fungus.

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**Introduction**

Peroxisomes are ubiquitously present in eukaryotic cells, and typically consist of a protein-rich matrix surrounded by a single membrane. They are involved in various metabolic processes, such as H₂O₂ metabolism and fatty acid β-oxidation, and some organism-specific biochemical reactions, such as the synthesis of cholesterol, bile acids and plasminogen in mammals, the glyoxylate cycle in plants, and methanol oxidation in yeast [1]. In mammals, defects in peroxisomes result in a variety of developmental defects; the most severe of which is Zellweger syndrome, which can cause death in early childhood [2,3].

The biogenesis of peroxisomes originates in the endoplasmic reticulum (ER) and consists of: (i) formation of the peroxisome membrane including acquisition of integral peroxisomal membrane proteins (PMPs); (ii) import of peroxisomal matrix proteins; and (iii) peroxisome proliferation [4]. Peroxisomes do not have their own DNA and therefore peroxisomal matrix proteins and PMPs are synthesized in the cytoplasm and imported into the organelles posttranslationally by complicated import machinery [5]. The proteins involved in peroxisomal import are designated as peroxins and their encoding genes are written as PEX [6]. The PEX genes were initially isolated in yeast models, and to date, more than 30 PEX genes have been identified in various organisms [1]. Homologues of most PEX genes are present in filamentous fungi [7–10].

Import of peroxisomal matrix proteins has been investigated intensively in different organisms, especially in yeast models. Most matrix proteins include a short peroxisomal-targeting signal (PTS), which can be either type PTS1 or PTS2 [4]. PTS1 is a tripeptide sequence (S/A/C) (H/R/K) (I/L/M) at the C terminus of the protein, and PTS2 is a nonapeptide sequence (R/K) (L/V/I) X₅(H/Q) (L/A) at the N terminus. PEX5 and PEX7 encode...
receptors that recognize and bind PTS1- and PTS2-containing proteins respectively [11–14]. PEX6 encodes an AAA type ATPase, and is involved in the import of peroxisomal matrix proteins by mediating the recycling of PTS receptors. In Aspergillus nidulans, Magnaporthe oryzae and Fusarium graminearum, PEX6 mutation results in mislocalization of PTS1- and PTS2-containing proteins, suggesting the involvement of PEX6 in both import pathways [10,15,16].

PMPs depend upon import pathways distinct to the PTS1- or PTS2-dependent import routes [17]. Most PMPs have one or more membrane protein-targeting signal (mPTS) consisting of a cluster of basic residues [18–20]. Pex19p is now known as both an import receptor and a chaperone for PMPs, which shuttles between the cytosol and peroxisomal membrane, binds and stabilizes newly synthesized PMPs in the cytosol, and is essential for PMP targeting and import [21,22]. Biophysical data indicate that Pex19p consists of a folded C-terminal and a flexible N-terminal sequence [23]. The C-terminal CAAX motif of Pex19p is essential for its farnesylation and ability to bind PMPs [24].

Peroxisomes in filamentous fungi were first characterized cytochemically in 1971, and peroxisomal biogenesis has been investigated intensively in recent years [25]. Research on peroxisomal biogenesis in filamentous fungi has revealed distinct features that were undetected in yeasts or mammalian cells [9,26–29]. An intriguing feature is their impact to pathogenicity of plant fungal pathogens. Disruption of PEX6 gene (CaPEX6) in anthracnose fungus Colletotrichum mangiferae (syn. C. orbiculare) damages peroxisomal metabolism and impairs infection severely, indicating the involvement of peroxisomal metabolism in pathogenicity of plant fungal pathogens [5]. This conclusion was reinforced by Δpex6 mutant in M. oryzae, which lacks appressorial melanization and fails in host penetration, and is therefore completely non-pathogenic [8,30]. PEX13 is required for appressorium-mediated plant infection by C. orbiculare [31]. PEX5 and PEX6 are critical to virulence and survival of F. graminearum on wheat [10]. In M. oryzae, the PTS1 pathway mediated by MoPEX5 and Pex19p, and PTS2 pathway mediated by MoPEX7 are both required for fungal pathogenicity, although the PTS1 pathway seems to play a predominant role [32,33]. Woronin bodies, which are fungus-specific organelles derived from peroxisomes, are also involved in pathogenicity of plant pathogens [34]. However, the characterized genes in fungal pathogens are all involved in the import of peroxisomal matrix proteins, whereas the import machinery of PMPs is still obscure.

M. oryzae is a heterothallic, haploid ascomycetous fungus that causes rice blast, the most destructive rice disease worldwide, and diseases on many other economically important cereal crops [35]. Infection with M. oryzae is initiated from the conidia, which are dispersed by splashing and tightly adhered to the hydrophobic leaf surface by an adhesive mucilage secreted on their tip [36]. Subsequently, the conidia germinate quickly and, within 8 h, form specialized infective structures called appressorium. The appressoria are darkly pigmented with melanin and accumulate high concentrations of glycerol to generate large internal turgor [37]. The appressoria attach to the leaf surface and immediately proceed with emergence of a slender hypha, the penetration peg, which ruptures the plant cuticle and cell wall to invade the underlying epidermal cells [38,39]. During appressorial matura-
tion and turgor generation, M. oryzae transfers abundant lipid bodies to the developing appressorium, coupled with rapid lipolysis. In caryokarys, lipolysis requires the metabolic processes in peroxisomes [30]. End product of peroxisomal lipolysis, acetyl-CoA, is also a major source for infection related morphogenesis of M. oryzae [30].

To understand better the molecular mechanisms of PMP import and their roles in fungal pathogenesis, we characterized MoPEX19, the ortholog of PEX19 gene in M. oryzae. Our data demonstrated that MoPEX19 plays crucial roles in peroxisomal maintenance and pathogenicity of the rice blast fungus.

### Materials and Methods

#### Fungal strains, growth conditions and transformation

M. oryzae wild-type Guy-11 [40] and all its derivative transformants and mutants strains were routinely cultured on complete medium (CM) [41] at 28°C for 3–14 days [42]. For genomic DNA isolation, mycelia were cultured in liquid CM for 3 days. Lipid medium, glucose medium and sodium acetate medium were prepared as described previously [33]. All fungal transformants were generated by *A. tumefaciens*-mediated transformation (JMT) as described previously [43]. CM plates containing 250 μg/mL hygromycin B (Roche, Mannheim, Germany), 200 μg/mL glucosinate–ammonium (Sigma, St Louis, MO, USA) or 800 μg/mL G418 (Sigma) and defined complex medium (DCM; 0.16% yeast nitrogen base without amino acids, 0.2% asparagine, 0.1% ammonium nitrate and 1% glucose, pH 6.0 with Na2HPO4) [8] containing 100 μg/mL chloramphenicol ethyl (Sigma) were used for screening corresponding transformants.

#### Table 1. The primers used in this study.

| Name   | Sequence (5′–3′) |
|--------|-----------------|
| 198TF  | AGGAAACAGGCAAGAAGTCT |  |
| 198TR  | CATCTTTTGACATCTATCCA |  |
| 19pyes2cds4 | CCCAAGGCTAAGGAGACCACCGCCGCCGACACA |  |
| 19pyes2cds5 | GCTCTGAATGACTTCTGAGCTTACTTGTAGTCTGA |  |
| HPH-BamHI | GGAGATCTGGGACTGCAACATCAAGACTGATT |  |
| HPH-Smal | CCCCCTGCCATCTCTTGTCTTGCTGCAG |  |
| 19uf | AATCAGTGGAAAGATGTTGCTG |  |
| 19dr | GCTCTAGATGGAAGAAAACCCCTGGAATACTT |  |
| 19ef | GGGGTACCCCAACGCTAACCAATCTCCACC |  |
| 19er | CCGAATTCGCTGTAAGATGTTGCTGAAG |  |
| 19ds2 | TCCCCCGGATGGGGCATCCCGGGCGGAGACA |  |
| 19ds3 | CGCGAAGCAGAAGAAGGATGCTGATTCGATGAAG |  |
| 1comup | CGGAAATCCGGACGGCGATGATGCTG |  |
| 1comdn | GCTCTAGAGGAGGAGAGGATGCTGAGCAGAAG |  |
| 19group | ATCGATGGTGAAGGTGATGCTGAC |  |
| 19prodn | GCTCTAGAGGGCCATCTTTGATGCTGCTG |  |
| GFP-Xb | GCCCTCTGAGGGCAGAAGGAGGAGGA |  |
| GFP-C-Sm | TCCCCGGGCGTACGATCCTGCTGCGTGG |  |
| NEO-Xh1 | CGGCTGAGGATGCTGTAAGATGTTGCTGAAG |  |
| NEO-Xh2 | CGCCTAGCTGAGGTAAGATGTTGCTGAAG |  |
| SUR-Xh1 | CGCCTAGATGGTGAAGGAGGATGCTGATTCGATGAAG |  |
| SUR-Xh2 | CGCCTAGATGGTGAAGGAGGATGCTGATTCGATGAAG |  |
| P47-cds2 | TCCCCCGGGATGGGGCATCCCGGGCGGAGACA |  |
| P47-cds3 | GCTCTAGATGGAAGAAGGATGCTGATTCGATGAAG |  |
| HexC-S4 | TCCCCCGGGATGGGGCATCCCGGGCGGAGACA |  |
| HexC-31-BstXI | CTCGAGAAGCAGAATGGCAGTATGCTGATTCGATGAAG |  |

*The restriction sites used were underlined.*

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Figure 1. Similarity of MoPEX19 homologs. (A) Amino acid sequences of HsPex19 (XP_501231.1) from humans, GzPex19 (XP_390112.1) from Gibberella zeae (F. graminearum), NcPex19 (XP_961091.1) from N. crassa, AfPex19 (XP_754525.1) from Aspergillus fumigatus, and ScPex19 (CAA98630) from S. cerevisiae were aligned with ClustalW. Identical amino acids are highlighted against a black background, conserved residues are shown on a
Bioinformatic analysis
The homolog of PEX19 in M. oryzae was identified by searching the Magnaporthe genome database [http://www.broadinstitute.org/annotation-genome/magnaporthe_comparative/MultiHome.html] with the protein sequence of Scpex19p from Saccharomyces cerevisiae (CA90630.1). Sequence alignments were performed using the mafft program [http://mofyle.pasteur.fr/cgi-bin/portal.py#forms:mafft], with the parameters Gap opening penalty of 2.0, offset value of 0.1. The resulting alignments were imported into the software GeneDoc 2.0 for type setting and into MEGA 5.0 to establish phylogenetic trees.

Nucleic acid manipulation and Southern blotting
The genomic DNAs were isolated using the cetyl trimethyl ammonium bromide method [41]. The restriction digestion, gel electrophoresis, ligation reactions, and PCR were all carried out using standard procedures. Southern blotting was performed using the digoxin high-prime DNA labeling and detection starter kit I (Roche) following the manufacturer’s instructions.

Total RNA was isolated using the Trizol reagent [Invitrogen, Carlsbad, CA, USA], and used as a template to synthesize cDNA using AMV Reverse Transcriptase [Takara Bio, Otsu, Japan]. The abundance of MoPEX19 transcripts was analyzed using primer pair 19RTF/19RTR on the 7500 Fast Real-Time System (Applied Biosystems, Foster, CA, USA) by calculating the average threshold cycle (Ct) value, which was normalized to that of Tubulin gene (MGG_00604). The relative abundance of gene expression at different developmental stages was compared with that in fresh conidia. Three replicates were performed for each sample.

Yeast complementation
The 1.1-kb fragment of MoPEX19 cDNA was amplified using the primer pair 19pyes2cdis4/19pyes2cdis5 and inserted into the SmaI/BstXI sites of the yeast expression vector pYES2 [Invitrogen] to generate pYES2-PEX19. pYES2-PEX19 was transformed respectively into S. cerevisiae wild-type strain BY4741 (MATa: his3A1 leu2A0 met15A0 ura3A0) and its derivative ScPEX19 (YDL065C) deleted mutant (Thermo Scientific, San Jose, CA, USA) using the lithium acetate method. To assess the ability of the transformants to utilize oleic acid, the yeast cells were precultured in liquid YPD medium (1% yeast extract, 2% peptone and 2% dextrose) to mid-log phase, washed three times with sterilized double distilled water, adjusted to OD600 = 1, and then grown on YNO medium [0.67% yeast nitrogen base with amino acids (Sigma), 0.1% oleic acid and 0.05% Tween 40, adjusted to pH 6.0] and SD medium [0.67% yeast nitrogen base with amino acids and 2% dextrose, adjusted to pH 6.0] in 5-μl aliquots of 10-fold serial dilutions at 30°C for 13–15 h.

MoPEX19 deletion and mutant recovery
For gene deletion, hygromycin phosphotransferase (HPH) gene expression cassette was amplified with primer pair HPH-BamHI/HPH-SmaI and inserted into SmaI/BamHI sites of pCAMBIA1300 (CAMBIA, Canberra, ACT, Australia) to generate p1300-KO. A 1.6-kb ResAl/XbaI upstream flanking sequence and 1.56-kb EcoR1/I/Kpnl downstream flanking sequence of MoPEX19 were amplified from M. oryzae genomic DNA with primer pairs 19uf/19ur and 19df/19dr respectively, and inserted into p1300-KO to generate the disruption vector pKO-MoPEX19, which was introduced into the M. oryzae wild-type. The candidates of gene deletion mutant were selected from hygromycin-resistant transformants by genomic PCR with primer pair 19cdis2/19cdis3 and confirmed by Southern blotting. Two of the confirmed mutants, Δmopex19-11 and Δmopex19-14, were selected for phenotypic analysis.

For mutant recovery, a 3.5-kb fragment containing the full length of MoPEX19 (1.2 kb), upstream (1.6 kb) and downstream (0.7 kb) sequences was amplified with primer pair 19comup/19comdn, and inserted into EcoR1/XbaI sites of p1300BAR [16] to generate complementary vector p1300BAR-19com, which was introduced into Δmopex19-14. The resulting glufosinate–ammonium-resistant transformants were picked up, from which the complemented transformants were selected by genomic PCR with primer pair 19cdis2/19cdis3 and confirmed by detecting the gene transcripts using quantitative PCR with primer pair 9RTF/19RTR. One of the confirmed transformants, Δmopex19-19com, was used in phenotypic analysis.

Figure 2. Complementation of Δscpex19 mutant by MoPEX19. S. cerevisiae wild-type (BY4741), Δscpex19, and transformants BY4741+pYES2, BY4741+MoEX19, Δscpex19+pYES2 and Δscpex19+MoEX19 were cultured on SD plates with glucose (A) or oleic acid (B). The expression of MoPEX19 restored the ability of Δscpex19 to grow on oleic acid. doi:10.1371/journal.pone.0085252.g002
Generation of fluorescent protein fusion constructs

To monitor the gene expression of MoPEX19, the 1.5-kb promoter region upstream MoPEX19 open reading frame (ORF) was amplified using the primers 19proup and 19prodn, and inserted into pBP5GFP [33] by PvuI/XbaI digestion to substitute the MoPEX5 promoter and generate the GFP expression vector p1300BP19GFP.

To track the cellular localization of MoPEX19, the coding sequence of GFP without termination codon was amplified with the primer pair GFP-Xb/GFP-C-Sma, and introduced into p1300BMGFP [16] by XbaI/SmaI digestion to replace the intact GFP ORF and produce p1300BMGFP-C. The coding sequence of MoPEX19 was amplified using the primer 19cds2/19cds3 from the total RNA as template, and introduced into p1300BMGFP-C by SmaI/BstXI digestion to produce the fusion vector p1300BMGFP-PEX19, in which the GFP–MoPEX19 fusion could be expressed under the control of the MPG1 promoter [41]. To mark the position of the peroxisomes, the fusion vector p1300NMRFPA containing RFP–PTS1 [16] was co-integrated into the p1300BMGFP-PEX19 transformants.

To investigate the localization of PTS1- and PTS2-containing proteins in wild-type, Δmopex19 mutant and complemented strains, the p1300BMGFP and p1300BMGFPB [16] were modified by replacing their hygromycin resistance gene cassette with the neomycin phosphotransferase II cassette to generate p1300NMGFPA and p1300NMGFPB, respectively. The ortholog of PMP47 (MGG_03247, assigned as MoPMP47 in present work) was used as a representative to assess the localization of PMPs. The MGG_03247 ORF was amplified with the primers P47-cds2 and P47-cds3, and introduced into p1300SMGFP-C, a vector derived from p1300BMGFP-C by replacing its BAR gene cassette with chlorimuron ethyl resistance gene (SUR), to produce the fusion vector p1300SMGFP-PMP47.

Figure 3. Sequential expression and cellular localization of MoPEX19. (A) Relative transcript abundance of MoPEX19 during appressorial development. Transcript abundance normalized to β-tubulin gene was measured by quantitative PCR at each time point and compared with that in the non-incubated conidia. (B) Fluorescent microscopy of co-transformants with GFP–MoPEX19 and RFP–PTS1 in hyphae, conidia and appressoria of Magnaporthe oryzae. The fluorescence of GFP–MoPEX19 was predominantly in the cytoplasm with some punctate enhancement, which partially overlapped with the red fluorescence of RFP–PTS1. Bar = 10 μm.

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Figure 4. MoPEX19 gene deletion and mutant complementation. (A) Diagram showing that the 1.22-kb MoPEX19 coding region was replaced by the 1.36-kb HPH cassette. An inner fragment within the deletion region was used as the probe for Southern blotting. Scale bar = 500 bp. (B) Total genomic DNA was isolated from the wild-type (lane 1), ectopic transformants (lanes 2 and 3), and potential Δmopex19 mutants (lanes 4–8, Δmopex19-11, Δmopex19-14, Δmopex19-20, Δmopex19-32 and Δmopex19-44), digested with SacI and subjected to Southern blotting. A 3943-bp hybridization band was detected in the wild-type, whereas 4971-bp bands were present in the five potential mutants, consistent with the gene deletion events. Ectopic transformant generated two bands, one of which was in equal size to the wild-type. (C) Gene transcription analysis of wild-type (Guy-11), Δmopex19-11 and Δmopex19-44, and Δmopex19-com by quantitative PCR. MoPEX19 transcripts were detected in similar abundance in the wild-type and complemented strains, but were completely undetectable in the mutants.

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The woronin body major protein, Hex1 (MGG_02696), was fused with RFP to mark woronin bodies [34]. The coding sequence of RFP without termination codon was amplified with the primer pair RFP-Xb/RFP-C-Sm from p1300NMRFPA [16], and introduced into p1300NMRFPA by XbaI/SmaI digestion to produce p1300NMRFP-C. The coding sequence of MoHEX1 was amplified using the primer HexC-54/HexC-31-BstXI, and introduced into p1300NMRFP-C by SmaI/BstXI digestion to produce p1300NMRFP-HEX1 [41]. All the primers used in present work were listed in Table 1.

Analysis of conidial germination, appressorial formation, turgor pressure and penetration

Conidia harvested from 10-day-old CM plates were suspended at 1x10^5/ml. Aliquots (30 μl) of the suspensions were incubated on a plastic coverslip in a moist chamber at 28°C for 48 h.

Figure 5. Distribution of the peroxisomal matrix proteins and PMPs in Δmopex19 mutants. Wild-type, Δmopex19-44 and complemented strains were transformed with GFP–PTS1, GFP–PTS2 and GFP–PMP47, respectively. Conidia of the transformants were harvested from 5-day-old CM plates and detected using confocal fluorescence microscopy. In the wild-type, the GFP–PTS1, GFP–PTS2 and GFP–PMP47 were all observed in punctate pattern, whereas in Δmopex19, GFP fluorescence was dispersed in the cytoplasm. In the complemented strains, GFP fluorescence was recovered into punctate patterns. Bar = 10 μm. doi:10.1371/journal.pone.0085252.g005
Conidial germination and appressorium formation were examined at 2, 4, 6, 8, 12, 24, 48 h post-incubation.

The incipient-cytorrhysis assay was performed to measure appressorial turgor. Conidia in 20 \( \mu \text{l} \) droplets (2 \( \times \) 10^5/ml) were allowed to form appressoria on plastic coverslips for 48 h. Water surrounding the conidia was removed carefully and replaced with 20 \( \mu \text{l} \) 0.5–4.0 M glycerol. The number of collapsed appressoria was counted after 10 min. The experiments were replicated three times, and >200 appressoria were observed in each.

Tolerance to \( \text{H}_2\text{O}_2 \), methyl viologen and Congo red

To assess the vegetative growth under oxidative stress, 2.5 or 5.0 mM \( \text{H}_2\text{O}_2 \) or 1 mM methyl viologen were added to CM, and the colonial diameters cultured for 3–5 days were measured. Cell wall integrity was assayed by growing the strains on CM supplemented with 200 \( \mu \text{g/ml} \) Congo red for 5 days.

Pathogenicity tests and infectious structures observation

Two-week-old rice CO39 seedlings and 7-day-old barley ZJ-8 were used for pathogenicity tests with conidia harvested from 10-day-old CM plates. Suspensions of 2 \( \times \) 10^4 conidia/ml supplemented with 0.25% (w/v) gelatin were applied by spray inoculation as described previously [41]. In the inoculation on detached leaves, the 30-\( \mu \text{l} \) droplets of the conidial suspension or mycelial plugs (5 mm diameter) were placed on leaf sections, and the leaves were incubated at 28 °C in darkness for 24 h and then in light for 3 days. For inoculation of wounded leaves, the detached barley leaves were scraped with sandpaper to remove the cuticles. To observe infectious structures, the inoculated barley leaves were discolored with lactic acid, heated at 65 °C for 2 h, and examined microscopically.

Fluorescent microscopy and transmission electron microscopy (TEM)

The cellular localization of GFP and red fluorescent protein (RFP) fusions were detected under a Leica SP2 Confocal System (Mannheim, Germany), with excitation 488 nm, emission 520 nm for GFP and excitation 558 nm, emission 583 nm for RFP. Nile red staining and fluorescein diacetate (FDA) staining were performed as previously described [33], and the fluorescence

Figure 6. Peroxisome and woronin bodies were absent in \( \Delta \text{mopex19} \) mutants. (A) Ultrastructure of the wild-type and \( \Delta \text{mopex19} \) mutant. Hyphae and conidia from 7-day-old CM plates were analyzed by TEM. Peroxisomes, nuclei and mitochondria were detected in the wild-type (upper left), while the peroxisomes were absent in \( \Delta \text{mopex19} \) (lower left). Typically 3–5 woronin bodies were seen beside the intercellular septum in the wild-type (upper right), but were undetectable in \( \Delta \text{mopex19} \) (lower right). Bar = 0.2 \( \mu \text{m} \). (B) Fluorescent localization of GFP-PTS1 and RFP-HEX1 in the wild-type and \( \Delta \text{mopex19} \) mutant. Bar = 10 \( \mu \text{m} \).

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was observed under an Olympus Xa21 fluorescent microscope with filters adapted to excitation 495 nm and emission 520 nm. (Tokyo, Japan).

For TEM analysis, the conidia and mycelia were collected on CM plate cultured at 28°C for 3–14 days. The collected fungal mass was treated as described [44] and examined under a JEM-1230 electron microscope (JEOL, Tokyo, Japan).

Results

Identification of MoPEX19

To find the PEX19 homologs in M. oryzae, the 350-amino-acid protein sequence of S. cerevisiae Scpex19p (CAA98630.1) was used to search the Magnaporthe comparative database with the BlastP procedure. A hypothetical gene MGG_00971 exhibited the most similarity to Scpex19p, with 24% amino acid identity. cDNA sequencing confirmed that the ORF of MGG_00971 was 1221-bp long, with three introns and four exons, and encoded a polypeptide of 356 amino acid residues, which was completely consistent with the annotation in the genome database. The predicted protein sequence of MGG_00971 showed 62% amino acid identity to hypothetical Pex19p from Gibberella zeae (XP_390112.1) and 57% identity to hypothetic Pex19p from Neurospora crassa (XP_961091.1) (Fig. 1). MGG_00971 was thus regarded as the PEX19 homolog in M. oryzae, and assigned as MoPEX19.

MoPEX19 is a functional homolog of PEX19 in S. cerevisiae

To investigate whether MoPEX19 (MGG_00971) is a functional ortholog of PEX19, we tested the ability of MoPEX19 to complement S. cerevisiae pex19 mutant. MoPEX19 cDNA carried by pYES2 was introduced into Δsopex19 mutant derived from strain BY4741. Δsopex19 exhibited severe growth deficiency on oleic acid medium, while the MoPEX19 integration fully restored the growth of the mutant on oleic acid (Fig. 2), suggesting that MoPEX19 has a function similar to ScPEX19.

MoPEX19 was distributed in cytoplasm and newly formed peroxisomes

The abundance of MoPEX19 transcripts was analyzed by quantitative PCR, which showed that the transcription of MoPEX19 was upregulated during conidial germination and appressorial development, with a peak at the initial germinated conidia incubated for 2 h (Fig. 3A). We examined the expression of GFP under the MoPEX19 promoter, but no fluorescence was visible in hyphae, conidia or appressoria, indicating that MoPEX19 was expressed at a low level and its promoter was weak. To assess the subcellular distribution of Mopex19 protein, we analyzed the localization of GFP-MoPEX19 fusion controlled by the MPG1 promoter. Fluorescent microscopy showed that the green fluorescence of GFP-MoPEX19 in hyphae, conidia or appressoria was present predominantly in the cytoplasm and enhanced at small puncta (Fig. 3B). The puncta overlapped partially with the fluorescence of RFP-PTS1, which represented the location of peroxisomes. PEX19 was previously demonstrated functioning in initial stages of peroxisomal biogenesis before the import of matrix proteins [24]. We thus conclude that Mopex19p was localized in cytoplasm and newly formed peroxisomes.

Disruption of MoPEX19

To determine the roles of MoPEX19 in peroxisomal biogenesis and fungal pathogenicity in M. oryzae, targeted gene replacement was performed. The knockout vector pKO-MoPEX19 was introduced into wild-type strain Guy-11 (Fig. 4A). Fifty hygromycin-resistant transformants were selected and checked primarily by PCR. Based on the PCR results, five of the possible gene-deleted mutants and two random insertion transformants were selected randomly and confirmed by Southern blotting (Fig. 4B), which indicated that gene replacement events occurred truly in the five mutants. Two of the confirmed mutants, Δmopex19-11 and Δmopex19-44, were further tested by quantitative PCR, which ensured that the gene expression was completely removed (Fig. 4C). For mutant complementation, a genomic fragment containing full-length MoPEX19 was reintroduced into Δmopex19-44. The resulting transformants were selected primarily by

Figure 7. Growth tests to determine the ability of Δmopex19 to utilize lipids as sole carbon source. Wild-type, Δmopex19 and complemented strains were inoculated with a plug of mycelium on minimal medium (MM) supplemented with 0.5% (v/v) Tween 80, olive oil or 12 g/l sodium acetate and cultured at 28°C for 12 days. doi:10.1371/journal.pone.0085252.g007
genomic PCR, and potential complemented transformants of them were confirmed by checking the gene transcription (Fig. 4C). The two \( \Delta \text{mopex19} \) mutants (\( \Delta \text{mopex19-11} \) and \( \Delta \text{mopex19-44} \)) and one of the confirmed complemented transformants (\( \Delta \text{mopex19-com} \)) were used for phenotypic analysis.

Peroxisomal matrix proteins and PMPs were mislocalized in \( \text{MoPEX19} \) deleted mutants

To investigate the functions of \( \text{MoPEX19} \) in the import of the peroxisomal matrix proteins and PMPs, we assayed the cellular distribution of PTS1- and PTS2-containing proteins and Mopmp47 (representing the PMPs) in \( \Delta \text{mopex19} \) mutants. The GFP fusions GFP–PTS1, GFP–PTS2 and GFP–PMP47 were introduced into the wild-type Guy-11, \( \Delta \text{mopex19} \) mutants (\( \Delta \text{mopex19-44} \)) and complemented strain (\( \Delta \text{mopex19-com} \)), respectively and detected by laser scanning confocal microscopy. In the transformants derived from the wild-type, the GFP fusions with PTS1, PTS2 or Mopmp47 were all distributed in punctate patterns, indicating their proper peroxisomal localization. However, the localization patterns were totally changed in \( \Delta \text{mopex19-44} \), where GFP–PTS1, GFP–PTS2 and GFP–PMP47 were all distributed in the cytoplasm, suggesting that these proteins were unable to be imported into peroxisomes. The peroxisomal localization of the GFP fusions was recovered by reintroduction of \( \text{MoPEX19} \) (Fig. 5). These results indicate that \( \text{MoPEX19} \) is indispensable for the import of peroxisomal matrix proteins and PMPs into peroxisomes of \( \text{M. oryzae} \).

Peroxisomal structures and woronin bodies vanished in \( \text{MoPEX19} \) deleted mutants

To investigate the influence of \( \text{MoPEX19} \) deletion on peroxisomal structures, we analyzed the ultrastructure of the \( \Delta \text{mopex19} \) and wild-type by TEM. In the wild-type cells, round peroxisomes were detected, mainly in the periphery of the cells and differentiable to the mitochondria in shapes and sizes (Fig. 6). However, peroxisomes or peroxisome-like structures were not detectable in the \( \Delta \text{mopex19-44} \) mutant. These results indicated that \( \text{MoPEX19} \) was vital to peroxisome maintenance. We also found

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**Figure 8. Tolerance of \( \Delta \text{mopex19} \) to H\(_2\)O\(_2\) and methyl viologen.** Wild-type, \( \Delta \text{mopex19} \) and complemented strains were cultured on CM and CM supplemented with H\(_2\)O\(_2\) (A) or methyl viologen (B) at 28°C for 5 days. doi:10.1371/journal.pone.0085252.g008
that woronin bodies were absent in the Δmopex19 mutant, in contrast with the wild-type, in which they presented mainly adjacent to hyphal septa and with a smaller size than the peroxisomes (Fig. 6A). Further, fusion of RFP with the Woronin body protein Hex1 resulted in punctate distribution that predominantly overlapped with GFP–PTS1 in accordance with the expected position of Woronin bodies in wild-type hyphae [34].

In contrast, RFP–Hex1 and GFP–PTS1 were both dispersed in the cytoplasm of the Δmopex19 mutant (Fig. 6B). These data together indicated that the peroxisomes and woronins were absent in Δmopex19 mutant.
MoPEX19 deletion led to defects in lipid metabolism and tolerance of reactive oxygen species (ROS)

To assess the effects of MoPEX19 deletion to peroxisomal metabolism, we investigated the capacity of lipid utilization and ROS elimination of the mutants. On media with Tween 80, olive oil and sodium acetate as sole carbon source, the development of Δmopex19 mutants was decreased greatly compared with that of the wild-type and the complemented strain, indicating disruption of lipid metabolism in the mutants (Fig. 7). The capacity to eliminate ROS was examined by comparing the tolerance to H₂O₂ and methyl viologen. On media containing H₂O₂ or methyl viologen, mutant growth was significantly reduced compared with the wild-type and complemented strain (Fig. 8A and 8B). The results suggested that deletion of MoPEX19 affected lipid metabolism and resistance to ROS in M. oryzae.

Deletion of MoPEX19 suppressed aerial hyphal growth and conidiation

The Δmopex19 mutants showed reduced radial growth and aerial hyphal development. On CM, Δmopex19-11 and Δmopex19-44 formed flat and bald colonies, which were significantly smaller than those of the wild-type and complemented strains (Fig. 9). The colonial pigmentation of the mutants was also decreased compared with the wild-type and complemented strains. Along with the increase in culture time, cell death was seen in Δmopex19 mutants, which initiated from the colonial center and expanded towards the edge. This suggests that MoPEX19 is essential for the vegetative growth and colonial melanization in M. oryzae.

Conidial generation was measured after 10 days culture on CM. Conidiation of the Δmopex19 mutants was dramatically reduced by ~50-fold compared with the wild-type and complemented strains (Fig. 10A and 10B). Cell death was also found in the conidia of Δmopex19 mutants (Fig. 10C). Almost all the conidia of the wild-type and complemented strains were adapt to FDA staining and emitted green fluorescence, compared with only ~30% of those of the Δmopex19 mutants, demonstrating reduced conidial vitality in the mutants (Fig. 10D and 10E). These results indicated that MoPEX19 was required for conidial generation and viability of M. oryzae. The cell death in the hyphae and conidia of the mutants may have been triggered by accumulation of cellular ROS [10], consistent with their hypersensitivity to H₂O₂ and methyl viologen.

MoPEX19 was required for conidial germination and appressorial development and turgor generation

The rates of conidial germination and appressorial formation were both significantly reduced in the Δmopex19 mutants (Fig. 11A and 11B). Under light microscopy, the 24-h appressoria of Δmopex19 mutants were less pigmented than those of the wild-type, Δmopex19 and complemented strains cultured on CM for 7 days were examined by light microscopy. Bar = 50 μm. (B) Statistical analysis of conidia produced on CM culture at 28°C for 10 days. Means and standard errors were calculated from three independent repeats. Double asterisks indicate significant differences to the wild-type (P<0.01). (C) Normal (I) and nonviable conidia (II) of Δmopex19 under light microscopy. Bar = 10 μm. (D) Viable conidia stained with FDA emitted green fluorescence while the dead ones did not. Bar = 10 μm. (E) Statistical analysis of viability of conidia cultured on CM at 28°C for 10 days. Means and standard errors were calculated from three independent repeats (at least 150 conidia of each strain were measured for each repeat). Double asterisks indicate significant differences to the wild-type (P<0.01).

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type, corresponding to reduced colonial melanization. Cytoplasmic leakage was found during the germination and appressorial development of the \( \Delta \)mopex19 mutants, which arose from the initial germ tubes and increased subsequently (Fig. 11C). This leakage was maybe related to the absence of woronin bodies. Consistent with the defects in lipids metabolism, the lipid mobilization from the conidia to appressoria was blocked in the mutants. Fluorescent microscopy combined with Nile red staining visualized the residual lipids in the conidia of the mutants, whereas hardly in the wild-type conidia incubated for 24 h (Fig. 11D).

The defects in appressorial pigmentation, lipid mobilization to the appressoria, and cytoplasmic leakage may affect substance accumulation and turgor generation in the appressoria. We compared the turgor of \( \Delta \)mopex19 mutants and the wild-type and

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**Figure 11. Conidial germination and appressorial formation of \( \Delta \)mopex19.** The conidial germination rates (A) and appressorial formation rates (B) of \( \Delta \)mopex19, wild-type and complemented strains on hydrophobic surface were calculated. (C) Conidia and appressoria incubated for 12 h. The appressoria of \( \Delta \)mopex19 were less pigmented than those of the control strains, and cellular leakage occurred in the germ tubes and appressoria of \( \Delta \)mopex19. (D) Nile red staining showed that more lipid residues were present in the conidia and germ tubes of \( \Delta \)mopex19 incubated for 24 h. (E) Cytorrhysis assay to compare the appressorial turgor genesis of \( \Delta \)mopex19 and the controls. The 24-h appressoria were soaked in glycerol and the rates of cytorrhysis were calculated. Means and standard errors were calculated from three independent repeats (at least 100 conidia of each strain were measured for each repeat). Single asterisks indicate significant differences at \( P < 0.05 \) and double asterisks indicate \( P < 0.01 \).

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**Figure 12. Tolerance to CR to compare the integrity of the cell wall.** Wild-type, \( \Delta \)mopex19 and complemented strains were cultured on CM supplemented with 200 \( \mu \)g/ml CR for 5 days.

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complemented strains by incipient-cytorrhysis technique. Treatment with 1, 2 and 3 M glycerol led to appressorial collapse in the \textit{Dmopex19} mutants at significantly higher level than that in the wild-type and complemented strains (Fig. 11E). These data indicated that the \textit{MoPEX19} gene plays an important role in appressorial morphogenesis and turgor generation, which are the key factors in host penetration.

**Figure 13. Pathogenicity test of \textit{Dmopex19}**. (A) Spray-inoculation with conidial suspension (1 x 10^5 conidia/ml) of \textit{Dmopex19}, wild-type and complemented strains on 2-week-old rice cultivar CO39. The symptoms were recorded at 7 days post-inoculation. (B) Spray-inoculation with conidial suspension (2 x 10^6 conidia/ml) on 7-day-old barley cultivar ZJ-8. The symptoms were recorded at 4 days post-inoculation. Detached barley leaves inoculated with 5-mm mycelial plugs (C) or with 20-μl droplets of conidial suspensions (2 x 10^5 conidia/ml) (D) were cultured for 4 days. (E) Wounded barley leaves inoculated with 20-μl conidial suspensions (2 x 10^6 conidia/ml) were cultured for 4 days. (F) Droplet-inoculated barley leaves were sampled at 24 and 48 h post-inoculation, discolored, and examined under a light microscope. The conidia (C), appressoria (A) and invasive hyphae (IH) are marked. Bar = 20 μm.

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Deletion of MoPEX19 altered integrity of cell wall

The reduced melanization may weaken cell wall [43] and the cytoplasmic leakage also hints cell wall damage. To confirm whether MoPEX19 deletion altered the integrity of the cell walls, the tolerance of the ∆mopex19 mutants to Congo red (CR), a cell wall disrupting agent, was compared with that of the control strains. Cultured on medium supplemented with 200 μg/ml CR, the colonial diameters of the mutants were remarkably lower than those of the wild-type and complemented strains (Fig. 12), indicating that MoPEX19 played a role in the integrity of cell walls of M. oryzae.

MoPEX19 is required for pathogenicity

To determine whether the alteration of appressorial melanization, turgor generation and cell wall integrity destroyed pathogenicity of the fungus, we performed inoculation tests on both rice and barley seedlings. The 14-day-old rice cultivar CO39 and 7- day-old barley cultivar Zj-8 were inoculated by conidial suspensions. No symptoms developed on rice at 7 days post-inoculation or barley leaves at 3 days post-inoculation infected with the ∆mopex19 mutants, in contrast to those infected with the wild-type and complemented strains, which generated abundant typical lesions (Fig. 13A and 13B). After inoculation with conidial droplets or mycelia plugs, the ∆mopex19 mutants did not cause any symptoms on detached barley leaves (Fig. 13C and 13D). Even on the wounded leaves from which cuticles were removed, the ∆mopex19 mutants were still non-pathogenic (Fig. 13E). By histological microscopy, abundant invasive hyphae were visible on the inoculation sites of the wild-type and complemented strains, which expanded quickly in the host tissue. However, no penetration peg or invasive hypha was found on the leaves inoculated with the ∆mopex19 mutant up to 48 h post-inoculation (Fig. 13F). The results indicated that MoPEX19 was indispensable to fungal pathogenicity and the MoPEX19 deletion destroyed host infection of the rice blast fungus completely.

Discussion

An increasing number of recent studies have revealed the machinry of peroxisomal biogenesis in filamentous fungi, which make it a good model for research in this field [29,46]. Peroxisomal biogenesis has already been proved to be related to peroxisomal metabolism and maintenance of peroxisomal structures in C. lagenarium, F. graminearum and S. cerevisiae [9,10,30]. However, the previous studies focused only on the peroxins participating in the import of peroxisomal matrix proteins, while the mechanism of PMP import and its roles in fungal pathogenicity remained unclear. Here, we characterized MoPEX19, an essential factor in peroxisomal biogenesis, and demonstrated that Mopex19p was indispensable for PMP targeting and maintenance of the peroxisomes, and was required in the pathogenicity and multiple developmental processes of the rice blast fungus M. oryzae.

Role of MoPEX19 in peroxisomal membrane biogenesis

Although the involvement of PEX19 in peroxisomal membranes biogenesis was well demonstrated in mammalian and yeast models [21,47], the functions of PEX19 are poorly understood in filamentous fungi. In present work, we demonstrated that PMP47, a representative PMP, was localized in the peroxisomes of the wild-type strain of M. oryzae, but in the cytoplasm of ∆mopex19 mutants, indicating the role of MoPEX19 in PMP import. In most studies, Pex19p was thought to be a receptor or soluble chaperone that binds the PMPs and maintains their solubility in the cytosol, delivers them to the peroxisomes and facilities their insertion into peroxisomal membranes [48,49]. In such cases, PEX19 mutation led to cytosolic localization of PMPs and severe damage, even complete absence, of peroxisomal structures, as that in pex19 mutants of S. cerevisiae and H. polymorpha [21,50,51]. Consistent with its function as a soluble receptor and/or chaperone, Pex19p was primarily cytosolic and associated with the peroxisomal membrane [18,22,52]. However in Yarrowia lipolytica and Pichia pastoris, Pex19p seemed not to act as a PMP receptor, because Ypex19p was primarily located in the peroxisomes, and in ∆pex19 and ∆Pipex19 cells, peroxisome remnants were found and PMPs were detected on these peroxisome-remodelling structures [53,54]. In ∆mopex19 mutants, no peroxisome or peroxisome-like structures were detected, suggesting that MoPEX19 was indispensable for peroxisomal maintenance. Resulted from the peroxisomal disappearance, the peroxisomal matrix proteins (PTS1- and PTS2-containing proteins) were distributed cytosotically in ∆mopex19 mutants, as the situation in pex19 mutants of S. cerevisiae and mammalian [21,24]. Mopex19p was found mainly in the cytosol, with punctate enhancements that were possibly newly formed peroxisomes.

MoPEX19 recovered the ability of ∆mopex19 mutant to utilize lipids. These findings suggest that MoPEX19 has a similar function to that in S. cerevisiae rather than in Y. lipolytica or P. pastoris. Furthermore, the imperfect overlap of GFP-MoPEX19 with RFP-PTS1 also agreed with the function of MoPEX19 in PMP import, because PMP assembly usually occurred before the import of peroxisomal matrix proteins represented by RFP-PTS1 [34]. Taken together, our data confirm that Mopex19 acts as a PMP receptor or chaperone that plays key roles in peroxisomal membrane assembly and maintenance of peroxisomal structures in M. oryzae.

The defects of P. pastoris pex19 mutant were functionally complemented by H. polymorpha PEX19 [51]. The human HsPEX19 recovered the import of peroxisomal matrix proteins and PMPs in pex19-mutated Chinese hamster ovary cells [24]. However, the ∆scepex19 mutant was not complemented by intact human PEX19 gene [21]. These results indicated that PEX19 is functionally interchangeable between closely related species but not conserved between yeasts and mammals. Our data showed that MoPEX19 complemented the growth defects of the ∆scepex19 mutant on oleic acid, implying that the functional conservation of Pex19p can span the evolutionary distance between filamentous fungi and yeasts. Human Pex19p acquired the complementary ability to ∆scepex19 mutant when its N-terminal region is substituted by that of Scpex19p, indicating that the N- and C-terminal regions of Pex19p have distinct functions and exhibited different conservation between organisms [21]. It will therefore be interesting to investigate the functions and conservations of different parts of Mopex19p in future.

Influence of MoPEX19 on peroxisomal metabolism and fungal development

Peroxisomes contain >50 enzymes involved in diverse metabolic processes, typically, fatty acid β-oxidation, glyoxylate cycle, and H2O2 elimination [1]. β-Oxidation is confined to the peroxisomes in yeast and plants, whereas it is present in both peroxisomes and mitochondria in mammalian cells [55–57]. In filamentous fungi, β-oxidation had been traditionally thought to occur exclusively in peroxisomes, until the discovery of mitochondrial β-oxidation led to acceptance of diverse β-oxidation pathways [58–60]. ∆mopex19 could not grow on fatty acids as sole carbon source, same as described previously in ∆mopex3 and ∆mopex6 [8,30,33], suggesting strongly that fatty acid β-oxidation in M. oryzae takes place predominately or exclusively in peroxisomes. Similarly, disruption of PEX6 disrupted growth of C.
logenarium on oleic acid, and *pex* mutants of *A. nidulans* exhibited severe reduction in fatty acid utilization [9,15]. However in *F. graminearum*, fatty acid utilization was slightly affected by *MoPEX5* and *PEX6* deletion, reflecting the offset of mitochondrial β-oxidation [10]. These results indicate that peroxisomal and mitochondrial β-oxidation are both present in filamentous fungi and the proportions of the two metabolic pathways are species specific.

Most of the enzymes involved in the glyoxylate cycle are located in peroxisomes in yeasts and mammals [61,62]. The Δ*mopex19* mutants lost the ability to utilize acetate, reflecting disorders in the glyoxylate cycle and suggesting that the cycle in *M. oryzae* occurs in peroxisomes. This agrees with previous studies on Icl1p, a core enzyme in the glyoxylate cycle. Icl1p was localized in the peroxisomes in *C. lagenarium*, and disruption of *ICL1* gene led to the incapability of *M. oryzae* and *C. lagenarium* to grow on acetate [63,64]. Furthermore, deletion of carnitine acetyltransferase, which mediates the movement of acetyl-CoA from cytoplasm to peroxisome, also prevented acetate utilization in *M. oryzae*. Surprisingly, the mutants *MoPEX5*, *MoPEX6* and *MoPEX7* all grew normally on acetate [8,30,32,33]. This may suggest the presence of novel import machinery for matrix proteins, which translocates the enzymes for the glyoxylate cycle into peroxisomes in a manner independent of *PEX5*, *PEX6* and *PEX7*. Consistent with this hypothesis, neither typical PTS1 nor PTS2 are found in Icl1p [64].

The end product of fatty acid β-oxidation, acetyl-CoA, together with glycolysis end products, can serve as energy donors that enter the citric acid cycle to produce ATP, or as constituents for synthesis of cellular components. Acetyl-CoA can be metabolized via the gluconeogenesis pathway to generate glycerol, which is the main solute in appressoria and indispensable for turgor generation [39]. The appressorial turgor of *Δmopex19* was dramatically reduced, as well in other *pex* mutants of *M. oryzae* and *C. lagenarium* [8,30,32,33,42], indicating that the acetyl-CoA-derived from β-oxidation contributes largely to appressorial glycerol generation. Acetyl-CoA can also be a starter unit of melanin, which could be the reason for why *Δmopex19* mutants showed decreased melanization in colony and appressoria. The shortage of appressorial melanization, which is crucial for maintenance of the cell wall strength and impermeability to glycerol [65], led to severe cellular leakage in germ tubes and appressoria of *Δmopex19*. Thus, the decrease in appressorial turgor is a synergy of decreased synthesis and leakage of glycerol, and the two aspects could result from the shortage of acetyl-CoA caused by insufficient fatty acid conversion. In addition, the cellular leakage reminds us of the function of woronin bodies, peroxisome-derived organelles that plug the septum pores when hyphae are damaged [34]. As expected, the woronin bodies could not be detected in *Δmopex19*.

Deficient fatty acid β-oxidation resulted in disordered appressorial turgor generation, which was crucial for fungal penetration. However, it seems not to be the sole reason leading to failure of infection with *Δmopex19*, because adding exogenous carbon did not recover the pathogenicity. Accordingly, only partial recovery of pathogenicity was found in other *pex* mutants by adding glucose or intermediate compounds of β-oxidation and glyoxylate cycle [9]. In *F. graminearum*, which does not form appressoria, the *PEx* genes still play a crucial role in pathogenicity [10]. All these facts hint that other major metabolic pathways in peroxisomes contribute to the fungal pathogenicity. ROS scavenging is another peroxisomal metabolism and the peroxisomal disorder enhanced ROS accumulation in yeast [66-68]. A peroxisome-associated protein,TmpL, is involved in intracellular ROS regulation in *Alternaria brassicola* and *F. fumigatus* [69]. In Δ*pex5* cells of *F. graminearum* and *M. oryzae*, ROS tolerance was reduced and cell death occurred [10,33]. In present work, the growth of *Δmopex19* was markedly reduced in ROS-containing media. The viability of its mycelia and asexual spores decreased, as seen in Δ*pex5* and Δ*pex6* mutants of *M. oryzae* and *F. graminearum*, which was thought to be triggered by redundant cellular oxidative stress [10,70]. These results demonstrate that the involvement of *MoPEX19* in ROS scavenging. However, mitochondria are also major organelles for ROS metabolism, which were demonstrated crucial in ROS neutralization and post-penetration growth in *M. oryzae* [71]. Disruption mitochondrial enoyl-CoA hydratase, Ech1, enhanced the sensitivity to oxidative stress and reduced the host penetration of the fungus. The peroxisomes and mitochondria are thus both required in regulating the ROS levels in filamentous fungi. The reduction of ROS scavenging in *Δmopex19* is likely another reason for its infection failure, because ROS produced by the host cells is a main barrier for fungal invasion and establishment of parasitism.

In *M. oryzae*, three *PEx* genes have been characterized to date: *MoPEX5*, *MoPEX6* and *MoPEX7*. *MoPEX5* contributes to import of PTS1 matrix protein; *MoPEX7* is involved in PTS2 matrix protein import; and *MoPEX6* participates in both PTS1 and PTS2 import [8,30,32,33]. Here, we demonstrated that *MoPEX19* contributed most among the three genes to peroxisomal biogenesis, because it influenced the import of both matrix proteins and PMPs. This led us to propose that *MoPEX19* plays more roles in peroxisomal metabolism, fungal development, and host invasion. According to our data, more severe metabolic defects were found in the Δ*mopex19* mutants, such as the incapacity of acetate utilization and cellular leakage, which were not observed in the other reported *pex* mutants. However, the development and pathogenicity of *Δmopex19* did not differ markedly to those of *Δmopex5* and *Δmopex6* [8,30,33]. This confirmed our previous conclusion that PTS1 contributes predominantly to fungal development and pathogenicity [33]. The peroxisomes play essential roles in the sexual reproduction of *F. graminearum* and *Podospora anserina* [10,26], but the sexual generation is unaffected in *Δmopex19* and any other *pex* mutant of *M. oryzae* [8,30,32,33]. This reflects the dissimilarity of the roles played by peroxisomal metabolism between fungal species.

In summary, as a key factor in peroxisomal biogenesis, *MoPEX19* participated in import of PMPs. Deletion of *MoPEX19* blocked the transport of PMPs from cytoplasm to peroxisomes, which led to complete disappearance of peroxisomal structures and improper localization of peroxisomal matrix proteins. The disorder in peroxisomal biogenesis disturbed severely peroxisomal metabolism, including fatty acid β-oxidation, the glyoxylate cycle, melanization, and cell wall synthesis. The metabolic defects led to multiple abnormalities of fungal development and resulted in loss of pathogenicity.

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**Author Contributions**

Conceived and designed the experiments: JW FL GS. Performed the experiments: LL ZZ ML. Analyzed the data: LL JW HJ. Contributed reagents/materials/analysis tools: RC XM HQ. Wrote the paper: JW LL YW.
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