PTS1 Peroxisomal Import Pathway Plays Shared and Distinct Roles to PTS2 Pathway in Development and Pathogenicity of *Magnaporthe oryzae*

Jiaoyu Wang¹, Zhen Zhang¹, Yanli Wang¹, Ling Li¹, Rongyao Chai¹, Xueqin Mao¹, Hua Jiang¹, Haiping Qiu¹, Xinha Du¹, Fucheng Lin², Guochang Sun¹*

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

**Abstract**

Peroxisomes participate in various important metabolisms and are required in pathogenicity of fungal plant pathogens. Peroxisomal matrix proteins are imported from cytoplasm into peroxisomes through peroxisomal targeting signal 1 (PTS1) or peroxisomal targeting signal 2 (PTS2) import pathway. *PEX*5 and *PEX*7 genes participate in the two pathways respectively. The involvement of *PEX*7 mediated PTS2 import pathway in fungal pathogenicity has been documented, while that of PTS1 remains unclear. Through null mutant analysis of *MoPEX5*, the *PEX*5 homolog in *Magnaporthe oryzae*, we report the crucial roles of PTS1 pathway in the development and host infection in the rice blast fungus, and compared with those of PTS2. We found that *MoPEX5* disruption specifically blocked the PTS1 pathway. *ΔmopeX5* was unable to use lipids as sole carbon source and lost pathogenicity completely. Similar as *ΔmopeX7*, *ΔmopeX5* exhibited significant reduction in lipid utilization and mobilization, appressorial turgor genesis and H$_2$O$_2$ resistance. Additionally, *ΔmopeX5* presented some distinct defects which were undetected in *ΔmopeX7* in vegetative growth, conidial morphogenesis, appressorial morphogenesis and melanization. The results indicated that the PTS1 peroxisomal import pathway, in addition to PTS2, is required for fungal development and pathogenicity of the rice blast fungus, and also, as a main peroxisomal import pathway, played a more predominant role than PTS2.

**Introduction**

Peroxisomes are single membrane-bound organelles present in almost all eukaryotes [1]. These organelles contain more than 50 different enzymes involved in various metabolisms, such as fatty acid β-oxidation, glyoxylate cycle and degradation of reactive oxygen species (ROS) [2,3]. Some special biochemical reactions, such as penicillin biosynthesis in *Penicillium* species, methyl alcohol utilization in yeasts and photorespiration in plants, also rely on peroxisomes [3,4,5]. The significance of peroxisomes in humans was demonstrated by diseases due to peroxisomal biogenesis disorders (PBDs), such as the Zellweger syndrome, the neonatal adrenoleukodystrophy, and the infantile Refsum’s disease [6].

Peroxisomes do not have their own internal DNA molecules [7,8]. Their matrix proteins and membrane proteins have to be encoded by nuclear genes, synthesized in cytoplasm and translocated to the organelle via post-translational transport [1]. The import machinery of the peroxisomal proteins consists of another group of proteins, named peroxins, which are encoded by *PEX* genes. To date, dozens of the *PEX* genes have been identified in different eukaryotes [8]. Mutations of these genes usually alter the size and number of peroxisome or misallocate the peroxisomal proteins [9,10,11]. To be recognized by the import machinery, the peroxisomal matrix proteins usually contain specific conserved motifs known as peroxisomal targeting signals (PTSs). PTSs fall into at least two categories, PTS1 and PTS2. PTS1 is conserved tripeptide SKL or its derivative (S/C/A-K/R/H-L) at C-terminal, which presents in most of the known peroxisomal matrix proteins [12]. PTS2 has a consensus (R/K)-(L/V/I)-X$_5$-(H/Q)-(L/A) located mainly at the N-terminus of a small amount of peroxisomal matrix proteins [13]. *PEX*5 and *PEX*7 genes encode the receptors for PTS1 and PTS2 respectively, which bind the peroxisomal matrix proteins directly or indirectly via co-receptors [14,15]. *PEX5* was found to have cross-talking to *PEX7* during PTS2 import in plants and mammals [15,16,17,18,19,20]. In recent years, much progress was made on the study of peroxisomal biogenesis in filamentous fungi [21,22,23,24]. And the involvement of peroxisomes in host invasion of plant pathogenic fungi was also demonstrated in several species [23,25,26,27].

*Magnaporthe oryzae* is a well-known pathogenic fungus that causes rice blast, one of the most devastating rice diseases. To penetrate the host surface, the fungus differentiates a well-specialized cell structure, appressorium. The appressoria are equipped with melanized cell wall and highly concentrated glyceral to generate enormous turgor [28]. Relying on the turgor, the fungus punches...
the host cuticles, penetrates into the cells, and subsequently grows within the host tissues.

Stored lipids in conidia are one source for the glycerol accumulation and are indispensable for the appressorium mediated infection [29]. During the pre-penetration stage, the lipids were degraded rapidly and migrated from germinated conidia into appressorium. In fungi, peroxisomes are the main location for lipid degradation [3]. The involvement of peroxisomal metabolism in the pathogenicity of M. oryzae was demonstrated by studies of PEX5 and PEX7 genes [23,25,30]. Disruption of PEX7 blocked the PTS2 import pathway in M. oryzae [30], pex7 null mutant exhibited peroxisome related defects such as reduced lipid degradation, and lost the ability to cause disease on rice [30]. These data fully demonstrated the requirement of PTS2 pathway in fungal pathogenicity. PEX6 gene encodes an AAA ATPase required in the recycle of PTS receptors [31], pex6 null mutant of M. oryzae lost the pathogenicity on rice and barley completely [30]. And further, the disruption of PEX6 seemed to make more damages to the fungus than that of PEX7 in conidiation, appressorial morphogenesis and melanization.

PEX6 was previously demonstrated to be required in both PTS1 and PTS2 pathways [32], the differences in the pex6 and pex7 null mutants therefore hinted a possible role of PTS1 pathway in fungal pathogenicity. However, the contributions of PTS1 pathway to fungal development and fungal pathogenicity remain unclear in any plant pathogenic fungus so far. To clarify the functions of PTS1 pathway, we characterized the PEX5 gene in M. oryzae (assigned as MoPEX5) by targeted gene replacement and compared it with MoPEX7. Our data showed that MoPEX5 participated in PTS1 pathway in M. oryzae and play multiple roles in vegetative growth, conidiation, appressorial morphogenesis, H2O2 resistance and pathogenicity. Further, the findings let us propose that PTS1, as a main peroxisomal import pathway, play a more predominant role than PTS2 to fungal development and pathogenicity in the rice blast fungus.

Materials and Methods

Strains, Growth Condition and Transformation

M. oryzae wild-type strain Guy11 [33], transformants and mutants were grown routinely on complete medium (CM) [34] using standard procedures [35]. The carbon starvation (CM-C) and nitrogen starvation media (CM-N) were prepared as described [34]. Lipid medium was made by adding 0.5% (v/v) olive oil or Tween 80 into CM-C [34]. Glucose and sodium acetate medium were CM-C supplied with 6 mM glucose or 50 mm sodium acetate, respectively. For liquid cultivation, the conidia in 1 x 1011/ml were shaken at 150 rpm in liquid CM at 28°C. The Agrobacterium tumefaciens-mediated transformation (ATMT) was performed as described [36]. CM plates containing corresponding antibiotics (250 μg/ml hygromycin B (Calbiochem, Germany), 200 μg/ml g418 (Sigma, USA) or 800 μg/ml g418 (Sigma, USA) were used in screening transformants.

cDNA Cloning of MoPEX5 and MoPEX7

The cDNA fragment of MoPEX5 and MoPEX7 were amplified from a cDNA library constructed with RNA of M. oryzae Guy11 [37] using primer-pairs p5cds3-Xb/P5cds4-Bm, and P7cds3-Xb/P7cds4-Bm respectively. The amplicons were cloned into pGEM-T easy vector (Promega, USA) and sequenced by Invitrogen, USA. The coding information of the sequences was analyzed at the GENSCAN web server at MIT (http://genes.mit.edu/GENSCAN.html) and the protein phylogenies were analyzed by using MEGA 5.0 software.

Construction of Gene Disruptants, Recovery Transformants and Fluorescent Transformants

Construction of the gene disruption vectors (p1301XGG-PEX5 and p1301XGG-PEX7), mutant screening and verification by PCR amplification and Southern blotting were described previously [32]. The selected mutant candidates were further confirmed by reverse transcription PCR using the primer pairs p9RTcheck1/p9RTcheck2 (for MoPEX5) and p7RTcheck1/p7RTcheck2 (for MoPEX7). One mutant strain of each gene, which showed typical phenotypes, was selected for mutant complementation. The 4.6-kb fragment containing full length MoPEX5 coding region and 1.5-kb upstream was amplified using primer pair P5f-Xb/P5r-Sc and cloned into pCR-XL-TOPO vector (Invitrogen, USA) and sequenced by Invitrogen, USA. To generate pTOPO-pex5, the 3.5-kb PCR fragment containing MoPEX7 coding region and 1.5-kb upstream was amplified using primer pair P7f-Xb/P7r-Ec and cloned into pCR-XL-TOPO to generate pTOPO-pex7. The XbaI-SacI digested MoPEX5 and XbaI-EcoRI digested MoPEX7 fragments were inserted respectively into the
Figure 1. Predicted amino acid sequence and phylogenetic analysis of MoPex5. (A) Alignment of predicted amino acid sequence of MoPex5 and its homologues. The amino acid sequences of GzPex5 (EAA686840) from Gibberella zeae, NcPex5 (EAA36111) from Neurospora crassa, AnPex5 (CBF85028) from Aspergillus nidulans, PcPex5 (AY366189) from Penicillium chrysogenum and ScPex5 (CAAB9730) from Saccharomyces 

---

MoPex5 : 

GzPex5 : 

NcPex5 : 

AnPex5 : 

PcPex5 : 

ScPex5 : 

---

Figure 1. Predicted amino acid sequence and phylogenetic analysis of MoPex5. (A) Alignment of predicted amino acid sequence of MoPex5 and its homologues. The amino acid sequences of GzPex5 (EAA686840) from Gibberella zeae, NcPex5 (EAA36111) from Neurospora crassa, AnPex5 (CBF85028) from Aspergillus nidulans, PcPex5 (AY366189) from Penicillium chrysogenum and ScPex5 (CAAB9730) from Saccharomyces.

B

MoPex5

NcPex5

GzPex5

AnPex5

PcPex5

ScPex5
corresponding sites in p1300BAR, a binary vector provided with glufosinate-ammonium resistance [32], to generate complementation vectors p1300BAR-MoPEX5 and p1300BAR-MoPEX7. Then the two complementation vectors were introduced into corresponding mutants respectively via AtMT. The transformants were selected and verified by PCR and Southern blotting. Strains with single-copy integration were selected and analyzed. To monitor the gene expression, the 1.5 kb promoter regions of MoPEX5 and

cerevisiae were aligned with Clustal W. Identical amino acids are highlighted against a black background, conserved residues are shown on a dark gray background, and similar amino acids are shown on a light gray background. Four probable TPR domains of MoPex5 were indicated with a line on the top of the sequence. (B) Phylogenetic relationship among MoPex5 and its homologues. The phylogenetic trees of the amino acid sequences were created using the MEGA 5.0 program according to the result of alignment.

doi:10.1371/journal.pone.0055554.g001

Figure 2. Mutation of MoPEX5 blocked the PTS1 peroxisomal import pathways and affected the lipid utilization. (A) The peroxisomal localization of the RFP-PTS1 and GFP-PTS2 in the wild type, Δmopex5 and Δmopex7. Conidia of the transformants Guy11/RFP-PTS1::GFP-PTS2, Δmopex5/RFP-PTS1::GFP-PTS2 and Δmopex7/RFP-PTS1::GFP-PTS2 harvested from 8-day-old CM plates were observed under a confocal fluorescence microscopy. (B) Lipid utilization of the wild type, Δmopex5 and Δmopex7. The Strains were cultured on medium with glucose, olive oil or Tween 80 as sole carbon source for 12 d at 28°C.

doi:10.1371/journal.pone.0055554.g002
Figure 3. Host infection of the wild type, Δmopex5 and Δmopex7. (A) The symptoms on the rice cultivar CO39, Brachypodium distachyon Bd21 and Barley ZJ-8 after spray inoculation with conidial suspension in $2 \times 10^4$ and $2 \times 10^5$ conidia/ml and the for 5 d. (B) The inoculated barley leaves were discolored, stained with aniline blue and observed under a fluorescent microscope. C, conidia; A, appressoria; IH, infection hyphae. The four-pointed star indicated hyphae that passed through the cell wall and invaded the neighbor cell. (C) The frequencies of penetration pegs formed by the appressoria after 24 h and 36 h on barley leaves were statistically compared.

doi:10.1371/journal.pone.0055554.g003
MoPEX7 were amplified by using the primer pairs P5 Pf P5 Pf-Px and P7 Pf-Px/P7 Pf-Px respectively. The amplifications were then inserted into pBMGF [32], a vector containing glufoximate-ammonium resistance and GFP expression cassette under MPG1 promoter, by Pst I/XhoI digestion, to replace the promoter and generate pBP5GFP and pBP7GFP. The pBP5GFP and pBP7GFP were integrated into Guy11 and resulted transformants were observed microscopically. For fluorescent col-localization, the RFP fusion vector p1300NMRFPA which carried G418 resistance, and GFP fusion vector pBARMGFPB which carried glufoximate-ammonium resistance [32], were integrated together into the wild type or mutant strains. The transformants were selected via the corresponding antibiotic and confirmed by PCR and Southern blotting. Of them, the single-copy integrated transformants were selected for further observation. All the primers used in present study were list in Table 1.

Nucleic Acid Manipulations and Southern Blot

Genomic DNAs were isolated by using the CTAB (hexadecyltrimethylammonium bromide) method [34] from 2-day-old mycelia in CM cultures shaken at 27°C, 150 rpm. Total RNA for cDNA amplification and reverse-transcription PCR was isolated by using Trizol reagent from mycelia treated in same way. Electrophoresis and restriction digestion were carried out using the standard procedures (Sambrook et al., 1989). Probe labeling and DNA hybridization were processed by using a digoxin labeling and detection kit with the procedure following the kit instruction (Roche, Germany).

Plant Infection Assays

For spray inoculation, 2 × 10^5 and 2 × 10^5 conidia/ml suspensions supplied with 0.25% gelatin and the seedlings of 18-day-old rice CO39, 6-day-old barley ZJ-8 and 22-day-old Brachypodium distachyon Bd21 were used. In each treatment, 4 ml conidia suspension was sprayed on 15 seedlings in one pot. Three replicates were set for each treatment. Inoculated plants were incubated in a controlled chambers first at 24°C and 24 h were tested. The turgor of 24 h appressoria was determined using the cytorrhysis/plasmolysis test. Conidial harvest, appressorium induction and appressorial turgor measurement were performed as described previously [39].

Fluorescent and Transmission Electron Microscopy

The fluorescence of FDA, Nile red and aniline blue were observed under fluorescent microscope Olympus Xa21 (Olympus, Japan). GFP or RFP fluorescence was observed using the Leica SP2 Confocal System (Leica, Germany). The objective was a 63× Plan- Apochromat (numerical aperture, 1.4) oil immersion lens. Images were taken through 488 nm wavelength laser excitation and 505–530 nm band pass emission filter. The hyphae and conidia harvested from 8-d-old CM plate and the appressoria incubated for 24 h were collected to perform TEM (transmission electronic microscopy). The electron microscope was JEM-1230 (JEOL, Tokyo, Japan). The samples treatment was processed as the procedure described [39].

Nile Red, FDA and H2O2 Assays

Nile red staining for conidia and appressoria was performed as described [39]. The viability of conidia was tested by incubating 1 × 10^5/ml conidia in 100 µg/ml FDA (fluorescein diacetate) solution for 3 min [40]. The viable conidia which could be stained and exhibited green fluorescence under a fluorescent microscope were counted and statistically analyzed. To assess the restraining of H2O2 to conidial germination, the 2 × 10^6 conidia were spread on a 90-mm CM plate and a 5-mm medium discs containing 0.5 M H2O2 was put on the center. The plates were incubated in 28°C for 3 days and the restraining rings formed were measured.

Results

Isolation and Disruption of MoPEX5

The putative homologues of PEX5 (MGG_10840) was identified by searching the Pex5p (CAA89730) of S. cerevisiae against M. oryzae genome using the blastP procedure [41], and assigned as MoPEX5. The cDNA amplicon showed that the open reading frame (ORF) of MoPEX5 was 2,044-bp long, contained 1 intron, and encoded a 650-aa peptide (MoPex5) which exhibited 57% aa identity to peroxin 5 of N. crassa (EAA36111) and 33% to that of S. cerevisiae (AAA64794) and contained four TPR domains (Figure 1A&B).

Previously, we deleted MoPEX3 and MoPEX7 respectively in the wild type strain Guy11 through A. tumefaciens-mediated transformation [42]. To determine the roles of MoPEX5, the Δmopex5 mutants were phenotypically analyzed. In view of the Δmopex7 mutant described from strain KJ201 [30], the Δmopex7 mutants from Guy11 were parallelly analyzed as a comparison with Δmopex5.

MoPEX5 Disruption Blocked PTS1 Peroxisomal Import Pathway

To investigate the functions of MoPEX5 in peroxisome biogenesis, the sorting of fluorescent proteins fused with PTS1 or PTS2 in Δmopex5 were tested. The RFP fused with a C-terminal consensus motif SRL [13] (RFP-PTS1) and GFP with an N-terminal PTS2 (GFP-PTS2) were co-introduced into Δmopex5, Δmopex7 and the wild-type respectively. The verified transformants were observed by a laser confocal microscope. In the transformants derived from the wild type strain, both the red and green fluorescence were detected predominantly as puncta at the peripheral region of the cells, indicating the proper peroxisomal localization of both PTS1 and PTS2 proteins (Figure 2A). In the transformants from the Δmopex5 strain, fluorescence of RFP-PTS1 was dispersed in cytoplasm while that of GFP-PTS2 remained punctate. In contrast, in the transformants from the Δmopex7 strain...
whose PTS2 pathway was blocked, RFP-PTS1 was seen punctate while GFP-PTS2 was cytoplasmic. Together with the related findings in yeasts and other fungal species [21,24,30], the result indicated that MoPEX5 gene, in contrast to MoPEX7, was involved PTS1 but not PTS2 peroxisomal import pathway.

Due to the peroxisomal defects, lipid utilizing in the ΔmoPEX5 was negatively affected (Figure 2B). On medium with lipids (olive oil or Tween 80) as sole carbon source, the growth of ΔmoPEX5 was abolished, in contrast to the efficient growth of the wild type strain. On glucose medium, ΔmoPEX5 grew normally, indicating its glucose utilization was unaffected. Further, ΔmoPEX5 remained the ability to use sodium acetate as carbon source (Figure S1). These data indicated that MoPEX5 was required in lipids metabolism in M. oryzae. And meanwhile, ΔmoPEX7 mutant was also deficient in lipids utilization.

MoPEX5 was Essential for Host Infection

To investigate the roles of MoPEX5 in plant disease, we performed pathogenicity test on rice, barley, and Brachypodium distachyon with two conidial suspensions (2×10⁴ and 2×10⁶ conidia/ml). At 4 days post inoculation (dpi), the leaves inoculated with the wild type conidia in either concentration developed large numbers of typical lesions that developed rapidly (Figure 3A). In contrast, ΔmoPEX5 at either conidia concentration produced no lesions on any host, indicating its pathogenicity was lost completely. As described, ΔmoPEX5 didn’t lead to symptoms on rice [30]. On barley and B. distachyon, at the lower conidial concentration, ΔmoPEX7 was also nonpathogenic. But when the conidial concentration was improved, typical lesions, although much less than those of the wild type, were generated on ΔmoPEX7 inoculated leaves of barley and B. distachyon. These results indicated that MoPEX5, as well as MoPEX7, was required to the pathogenicity of M. oryzae. And MoPEX5 was likely more contributual than MoPEX7.

The histological evidence of the pathogenic defects was found by microscopic observation of the infectious development on barley leaves (Figure 3B&C). At 24 h post inoculation (hpi), most of the wild-type appressoria (72.4%) had penetrated into the host cells and formed branched or still unbranched infectious hyphae. The infectious hyphae expanded rapidly in the infected cells in subsequent 6-10 hours and filled them up to 36 hpi. At 48 hpi, the infectious hyphae extruded their tips out of the first infected host cells and invaded the neighbor ones. The penetration ability of ΔmoPEX5 was lost completely. No efficient penetration was found on the leaves inoculated with ΔmoPEX5. The penetration ability of ΔmoPEX7 reduced remarkably. Most of its appressoria (88.3%) kept un-penetrated up to 24 hpi, and the typical infectious hyphae could not be found until 36 hpi. Meanwhile, the growth of infectious hyphae of ΔmoPEX7 was slowed down, which took about 12 h or more to fill the first infected cells. These data, corresponding with the result of pathogenicity test, indicated that the MoPEX5, as well as MoPEX7, was required in host infection, and this requirement likely related to both cuticle penetration and further development within host cells.

MoPEX5 Disruption Resulted in Reduced Vegetative Growth and Abnormal Colonial Morphology

To investigate the reasons for the penetration defects of ΔmoPEX5, possible pathogenicity-related phenotypes were analyzed. On complete media (CM), ΔmoPEX5 grew slower and formed sparse and fascicular aerial hyphae, in contrast to the wild type and ΔmoPEX7 (Figure 4A). On carbon or nitrogen starvation media, ΔmoPEX5 also exhibited reduced growth compared with the wild type and ΔmoPEX7 (Figure 4B). The melanization of ΔmoPEX5 colony was reduced on CM. This reduction was also found in liquid cultures. In liquid CM, the wild type and ΔmoPEX5 produced abundant melanin which made their cultures dark, while ΔmoPEX5 in same conditions exhibited much lighter color (Figure 4C). This result indicated that MoPEX5, unlike MoPEX7, was involved in vegetative growth and colonial morphology of M. oryzae.

MoPEX5 Disruption Reduced Conidial Genesis and Viability

The generation of conidia and conidiophores of ΔmoPEX5 strain reduced drastically (Figure 5A). On 9-day-old CM plates, ΔmoPEX5 produced 32.1±6.21 conidia per mm², equivalent to only 3.8% of that of the wild type (Figure 3B); ΔmoPEX7 produced 665.3±45.35 conidia per mm², reducing to 79.3% of the wild type. Moreover, the conidia of ΔmoPEX5 were nonviable in much higher proportion than those of the wild type and ΔmoPEX7. The nonviable conidia were easily distinguished by their incapability of FDA staining (Figure 5C). These nonviable conidia were either hollow with only shells or with granular residues inside their cells. The residues were capable of Nile red staining, indicating that their main components were lipids. Additionally, the proportion of the nonviable conidia of ΔmoPEX5 increased rapidly along the culture time (Figure 5D). On 6-day-old CM plates, 10 percent of ΔmoPEX5 conidia were nonviable, slightly higher than those of the wild type and ΔmoPEX7. Whereas on 14-day-old CM plates, the percentage of nonviable conidia of ΔmoPEX5 reached to 70%, in contrast to those of ΔmoPEX7 and the wild type which were still under 20%. The lower viability of spores was also found in pex5 mutant of Fusarium graminearum, which was related to the accumulation of ROS followed by necrotic cell death [44].

The less viability of ΔmoPEX5 conidia was resulted possibly from the defective lipids conversion. The conidial viability was also reflected in their germination. Harvested from 8-day-old CM plates and incubated on an inductive membrane for 24 h, 98% conidia of the wild type germinated, while only 77% of ΔmoPEX5 did (Figure 5E), corresponding with the result (76%) in FDA assay. ΔmoPEX7 exhibited a slight delay in germination at first 4 h but no final difference to the wild type at 24 h. We considered that the low conidial viability and the reduced germination was the first reason for the failure of ΔmoPEX5 in infectivity.

MoPEX5 was Required for Appressorial Formation and Morphology

Compared with the wild type and ΔmoPEX7, the ability of appressorial formation of ΔmoPEX5 was reduced significantly. Induced for 24 h, only 22% germinated conidia of ΔmoPEX5 formed appressoria, in contrast to the 97% of the wild type and ΔmoPEX7 (Figure 6A). Under the light microscope, the appressoria
Figure 5. Conidial generation and viability of Δmopex5, Δmopex7 and the wild type. (A) The development of the conidia and conidiophores of the strains grown on complete medium. (B) Statistical analysis of conidial production. The conidia produced by the strains grown on complete medium for 9 days were collected and counted. (C) Fluorescence staining of the conidia of the strains. The conidia were stained with fluorescein diacetate (FDA) and Nile red then observed under a fluorescent microscope. a, the viable conidia stained with FDA emitted bright green fluorescence while the dead ones did not; b, a viable conidium stained with FDA and Nile red; c and d, nonviable conidia stained with FDA and Nile red. (D) Percentages of nonviable conidia of the strains on complete media cultured for different time. (E) Germination rates of conidia of the strains harvested from 8-day-old complete medium and incubated on inducible membrane.

doi:10.1371/journal.pone.0055554.g005
of Δmopex5 could be found less pigmented than those of Guy11 (Figure 6B). For further confirmation, we performed the transmission electron microscopy (TEM). In 24 h appressoria of wild-type and Δmopex7, a distinct melanin layer was detected between cell wall and plasma membrane as described previously [23]. But in Δmopex5, this melanin layer was absent (Figure 6C). Together with the lighter color of the colony and liquid culture of Δmopex5, these data indicated that MoPEX5 was involved in melanin synthesis in mycelia and appressoria.

Cell wall integrity is another key factor in addition to melanization to maintain appressorial function. To compare the cell wall integrity, the sensitivities of the strains to Calcofluor white (CFW), a cell wall-perturbing agent that binds to cellulose and chitin, were tested. Compared with the wild type, the sensitivity of Δmopex5 to CFW was increased (Figure 7A&B), indicating its cell wall was weakened. In addition, the tolerance of Δmopex5 to sodium dodecyl sulfate (SDS) was also reduced than Guy11, indicating a possible role of MoPEX5 in integrity of cell membrane (Figure S2). Taking together, we concluded that MoPEX5 was involved in the appressorial formation, appressorial melanization and integrity of cell wall and cell membrane; and the reductions of Δmopex5 in these aspects were the second reason for its infectional defects.

MoPEX5 Participate in Lipid Mobilization and Appressorial Turgor Generation

In the wild type conidia induced for 24 h, the conidial contents were degraded completely and transferred into the appressoria, and then the conidia turned hollow and wizened. While in Δmopex5, as well as Δmopex7, numbers of droplets remained in appressoria, germ tubes and conidia kept undegraded even after 48 h (Figure 6B). These droplets were capable of staining by Nile red, indicating they were undegraded lipids (Figure 8A). In contrast, only very weak fluorescence was detected in the wild type which presented only in appressoria but not in conidia or germ tubes. Taking together with the result of lipid utilization, we concluded that the lipids degradation and their mobilization from conidia to appressoria were blocked in Δmopex5, as well in Δmopex7 mutants.

Defective melanization, weakened cell wall and inadequate lipids conversion might influence the glycerol accumulation and turgor-genesis in appressoria. By using the cytorrhysis/plasmolysis test [28], we measured the appressorial turgor. Dipped in glycerol solutions, the appressoria of each strain exhibited both cytorrhysis and plasmolysis (Figure 8B). In the wild type, the plasmolysis occurred much less than cytorrhysis. However in Δmopex5, the percentage of plasmolysis was improved significantly, exceeding that of cytorrhysis greatly. This result, corresponding with its missing melanin layer, indicated that the appressoria of Δmopex5 lost the glycerol impermeability and failed to maintain the turgor. The appressoria of Δmopex7 remained the ability to arrest glycerol where cytorrhysis occurred slightly more than plasmolysis. Nevertheless, the ratio of plasmolysis/cytorrhysis in Δmopex7 was higher than that of the wild type, indicating the cell wall integrity of its appressoria was also reduced, although no visible difference was found in its melanin layer and CFW resistance. At each glycerol concentration, the total percentages of cytorrhysis-occurred appressoria plus plasmolysis-occurred ones of each mutant were higher than those of the wild type, indicating the glycerol accumulation and appressorial turgor were reduced in both mutants. These reductions were possibly resulted from the blockage of lipids degradation and mobilization. The appressorial turgor was related directly to fungal penetration, thus its reduction was regarded as the third reason for the infectional defects of Δmopex5, as well Δmopex7.
Glucose Supplement or Host Cuticle Removal Improved the Virulence of Δmopex5

The lipids reserved in conidia is an important source of appressorial melanin, cell wall and glycerol, therefore the infectional defects of Δmopex5 were possibly resulted from defects in the lipids conversion. To confirm this deduction, we tested the effects of additional carbon source to virulence of Δmopex5 and Δmopex7. Being supplemented with Glucose in conidial suspensions, both mutants offset their pathogenic defects partially. Δmopex5 generated visible lesions and Δmopex7 accelerated its symptom development (Figure 9A). Taking together, we concluded that the incomplete lipid degradation in Δmopex5 caused nutrition insufficiency, which led to the defective melanization, weakened cell wall and reduced glycerol accumulation, and finally resulted in the defects in cuticle penetration.

To learn whether the genes functioned also at post-penetration stage, droplet inoculation was conducted on wounded barley leaves whose cuticles were removed (Figure 9B). In contrast to the intact leaves where Δmopex5 didn’t cause any lesions, the wounded leaves inoculated with Δmopex5 generate slight symptoms. Also, Δmopex7 caused more advanced lesions on wounded leaves than on intact ones. This result indicated that the virulence defects of Δmopex5 and Δmopex7 were partially restored by removing the cuticles, corresponding with the involvement of MoPEX5 and MoPEX7 in cuticle penetration. However, despite on wounded leaves, the lesions derived from Δmopex5 or Δmopex7 were still less developed than those from the wild type. Combined with the histological observation of infection, the result indicated that MoPEX5, as well as MoPEX7, played roles in fungal development within plant tissue in addition to the cuticle penetration.

Δmopex5 and Δmopex7 Mutants were both Hypersensitive to H2O2

Since neither the glucose supplement nor the cuticle removal restored the full virulence of the mutants, the defects in lipids degradation and cuticle penetration were unable to fully explain the infection failure. In view of that ROS in host cells are a main barrier for fungal invasion while ROS degradation is another main
metabolism in peroxisomes, we tested the abilities of the strains to degrade H$_2$O$_2$, a main form of ROS. On CM plates, the conidial germinations were restrained by H$_2$O$_2$ and formed restraining rings. The rings formed by Δmopex5 and Δmopex7 showed no difference in between, but were larger significantly than that of the wild type (Figure 10A&B). Similarly, the H$_2$O$_2$ tolerance of Δmopex5 and Δmopex7 during vegetative growth were also reduced significantly in comparison with the wild type (Figure S3). These data indicated that the abilities of H$_2$O$_2$ degradation of Δmopex5 and Δmopex7 were reduced. The pathogens must overcome ROS in host cells to invade smoothly. The reduced ability of ROS degradation was thus a possible reason for why the mutants slowed down the growth of their infectious hyphae in host tissue and developed lower symptom despite on wounded leaves or with supplemented Glucose. Taking the phenotypic data together, we concluded that the infection failure of the mutants was resulted not only from the penetration defects caused by the insufficient lipid

Figure 8. The lipids mobilization and turgor genesis of the wild type, Δmopex5 and Δmopex7. (A) Numerous lipid residuals (indicated by the arrows) in the 24 h appressoria of Δmopex5 and Δmopex7 were visualized by Nile red staining. To improve the staining efficiency, 10 ppm tricyclazole was added to the conidial suspensions [65]. (B) Cytorrhysis and plasmolysis test of 24 h appressoria. The appressoria were soaked in gradient concentrations of Glycerol. The percentages of cytorrhysis and plasmolysis occured were counted and statistically compared. The error bars on the top of the white columns represent standard deviations of plasmolysis.

doi:10.1371/journal.pone.0055554.g008
degradation, but also the developmental defects in host tissue caused by the decrease resistance to ROS.

Gene Reintroduction Restored the Virulence of the Corresponding Mutants

To ensure that the pathogenicity-related changes in Δmopex5 and Δmopex7 were associated truly with the genes disruption events, the MoPEX5 and MoPEX7 genes were reintroduced into their respective mutants. Transformants carried a single-copy integration were selected and ensured by Southern blot analysis. The complementary transformants of MoPEX5 (P5R) and MoPEX7 (P7R) were all able to grow on the media with lipid as sole carbon source and exhibited full virulence (Figure 11A&B). We concluded that the deletion of MoPEX5 and MoPEX7 were responsible for the virulence defects and the related phenotypes in M. oryzae.

MoPEX5 and MoPEX7 were HighlyExpressed in Conidia and during Conidial Germination

In order to investigate the temporal and spatial patterns of MoPEX5 and MoPEX7 expression during infection-related development, 1.5 kb promoter fragments upstream of MoPEX5 and MoPEX7 protein-coding sequences were fused respectively to the green fluorescent protein-encoding gene GFP. The fusions were introduced into the wild type strain Guy11, and the transformants with a single integration of the plasmid were selected for fluorescent observation. As a control, a 1.6 kb promoter fragment with a single integration of the plasmid were selected for introduction into the wild type strain Guy 11, and the transformants, exhibited GFP fluorescence, but MoPEX5(p):GFP and MoPEX7(p):GFP were still in fairish levels. But subsequently, along with the swelling, melanization and maturation, the fluorescence was weakened incessantly and almost absent in 24 h mature appressoria, in contrast with those of MPG1(p):GFP which kept continuous high expression during this process. The results indicated that MoPEX5 and MoPEX7 had similar expression patterns, namely, a high expression in conidia and during germination and subsequent decline, which were also confirmed by quantifying the transcripts at each time points using quantitative-PCR (Figure S4). The expression patterns, corresponding with the number variation of peroxisome we previously described [32], hinted a high requirement of peroxisomal metabolism during conidial germination.

Discussion

The characterization of peroxisomal biogenesis related genes (PEXs) and peroxisomal metabolism related genes demonstrated that the peroxisomes are required in fungal pathogenicity [23,25,45]. However, the peroxisomal biogenesis machinery is so complicated that different genes in same organism or homologous genes in different species may play different roles [27,46,47]. Here, we focused on the shared and distinct contributions of MoPEX5, the predicted PTS1 receptor encoding gene, to the PTS2 receptor encoding gene, MoPEX7, in fungal development and pathogenicity of M. oryzae. The result indicated that MoPEX5 participated specifically in PTS1 pathway. Both MoPEX5 and MoPEX7 are required in multiple aspects in fungal development and pathogenicity, and comparatively, MoPEX5 played more roles.
oryzae genome. These data indicated that PEX5 and PEX7 function independently in ascomycetes.

The Involvement of Peroxisome in Fungal Infection Related to Lipids Metabolism and ROS Degradation

The Δmopex5 mutant, together with Δmopex7 and Δmopex6, demonstrated the involvement of the peroxisomes in multiple pathogenicity-related events, such as conidiation, appressorial morphogenesis and hyphal development in host tissue [23,25,30]. Peroxisomes participate widely in various metabolisms, of which, the lipids degradation and ROS removal are conserved among different organisms. As known, the conidia of M. oryzae have no exogenous nutrition to use and have to degrade their own storage reserves to supply all the material and energy for infection morphogenesis, while lipid is one of the main reserves and its importance to fungal infection has been demonstrated [29,57,58]. In eukaryotes, lipids are metabolized by two peroxisomal processes, β-oxidation and glyoxylate cycle, to produce acetyl-CoA [3]. Acetyl-CoA is an important molecule which can be used to generate ATP in TCA cycle or convert to glucose via gluconeogenesis. The resultant Glucose can be further converted to glycerol, as well chitin and β-1, 3-glucan, the components of fungal cell wall. Additionally, Acetyl-CoA is also the starter and extender units in fungal melanin synthesis. Thus, lipids are an important source for melanin, glycerol, cell wall and energy (ATP), the key factors for fungal infection. Δmopex5 and Δmopex6 exhibited defects in all these factors, and as well Δmopex7 in glycerol and cell wall [23,25,30]. And meanwhile, Δmopex5, Δmopex6 and Δmopex7 were defective in lipids utilization and in lipids mobilization from conidia to appressoria. Moreover, the supplemented glucose could partially offset the pathogenicity of the mutants. These facts indicated that the contributions of the peroxisome to fungal pathogenicity were related, to a great extent, to lipids degradation. Correspondingly, the MoPex5 and MoPex7 genes were highly expressed during the conidial germination and initial emergence of appressoria, which are just the main period of lipid degradation [25]. This deduction can also be confirmed by the mutations of the key genes in β-oxidation and glyoxylate cycle which caused some shared defects as the pex mutants to the fungus [23,45,58].

On the other hand, the host cells produce abundant ROS to suppress pathogen infection. The pathogens have to remove these excess ROS for survival. Δmopex5 and Δmopex7 showed slowed hyphal growth in host cells and meanwhile the decreased resistance to H₂O₂, implying that the reduction of ROS...
degradation was responsible, at least partially, for the defects in post-penetration phases. To analyze the roles of the enzymes in ROS degradation, such as catalase and peroxidase, in fungal infection, is thus interesting and will improve the understanding of this topic. In addition to lipids and ROS metabolism, the other peroxisomal metabolic reactions also have the possibility to affect the fungal pathogenicity, for example, a hypothetical multicopper oxidase containing PTS1 showed closely similarity to Abr1p, a protein involved in melanin biosynthesis in Aspergillus fumigatus [59].

PTSI and PTS2 Pathways Contribute Differently to Fungal Pathogenicity

Since the symptom is an accumulated result of pathogenicity-related factors, the levels of virulence decrease are determined by the numbers and the levels of the factors affected by gene deletion. MoPEx5 was involved in more pathogenicity-related factors than MoPEx7, thus ΔmopeX5 lost entirely pathogenicity while ΔmopeX7 did not. To find the clues to the predominance of MoPEx5 in pathogenicity, we investigated the PTSs presented in the key enzymes of lipids degradation. In the 33 hypothetic enzymes collected from M. oryzae genome, 16 contained typical PTSs, and of which, 13 were PTS1 and three were PTS2 (Table S1). The presences of both PTS1 and PTS2 in the enzymes corresponded to the requirement of both MoPEx5 and MoPEx7 in lipids metabolism. Meanwhile, PTS1 presented predominantly in the enzymes, implying that MoPEx5 probably contributed more to lipids metabolism than MoPEx7, although their difference was not detected in lipid utilizing experiment. This is maybe the reason for why more related phenotypes, namely, melanization, cell wall biogenesis and glycerol accumulation, were affected in ΔmopeX5, while in ΔmopeX7, only the glycerol accumulation. This phenomenon inferred that the fungus likely ensured its melanization preferentially to glycerol accumulation when facing to nutrient starvation. Additionally, some other PTS containing proteins in M. oryzae could make better understanding for the phenotypes of ΔmopeX5 and ΔmopeX7. For example, a PTS1- (SKL) containing protein, MGG05130, was a homologue of SPS19 which was required in the vegetative growth and sporulation of S. cerevisiae and N. crassa [60,61]. A multicopper oxidase, MGG07220, which presented a PTS1 (AKL), showed similarity to Ab1r1p, an enzyme involved in melanin biosynthesis [59]. This finding hinted a possibility that the melanization defects in ΔmopeX5 were derived also from melanin synthesis itself in addition to the nutrient starvation. That is maybe the reason for why ΔmopeX5 still lacked melanin in rich media (CM) and why ΔmopeX7 was unaffected in melanization despite its lipids metabolism was disordered.

But remarkably, more than a half of these enzymes had no PTS1 or PTS2. One possibility of this fact is that the metabolisms known as occurred dominantly in peroxisomes could also be performed in cytoplasm or other organelles; another possibility is that these enzymes has special PTS which could be recognized by PEX5, PEX7 or even novel receptors in import pathways unknown so far. The possibility of the existence of new import pathways was demonstrated by PEX20 in P. anserina which mediated the import of peroxisomal matrix proteins independently for some specific developmental processes [46].

To reveal the combined effects of MoPEx5 and MoPEx7, we tried double deletion but failed to obtain double mutants after screening more than 300 transformants. Thus, the double deletants of the two genes is maybe lethal in M. oryzae. Nevertheless, MoPEx6, which was involved in both PTS1 and PTS2 pathways, gave some clues to the synergic effects of MoPEx5 and MoPEx7 [23,25]. The ΔmopeX6 mutant lost the pathogenicity completely and exhibited slower vegetative growth, reduced appressorial melanization and decreased conidiation, resembling closely to ΔmopeX5, and thus indicating that the defects of ΔmopeX6 were related mostly to its disorder in PTS1 pathway. But the damages enhanced by the disorder of PTS2 pathway could also be found in ΔmopeX6 mutant. For example, the additional glucose offset the virulence of ΔmopeX5, despite in a low level, but failed to do that in ΔmopeX6. Thus, the phenotypes of ΔmopeX6 reflected in some extent the defects derived from disorder of both PTS pathways. Despite that, the phenotypes of ΔmopeX6 could not be simply regarded as a summation of ΔmopeX5 and ΔmopeX7, because no evidence so far indicated PEX6 could control all the functions of PEX5 and PEX7. Therefore, it would be very interesting to further clarify the mechanism and the roles of import of peroxisomal matrix proteins, including new ones such as that mediated by PEX20 [46], as well as the import of peroxisomal membrane proteins which was mediated by PEX19 [62].

In addition, some slight differences could be found between the MoPEx7 mutants derived from Guy11 and from KJ201 [30]. For example, the ΔmopeX7 from KJ201 exhibited dramatic decrease in conidiation while that from Guy11 did not. And some other quantitative differences were also found in between. Such differences derived from strain backgrounds could also be found when we compared the parallel studies of Δpth2, ΔmopeX6 and Δhex1 [23,25,63,64]. This reminds us that investigating a gene in more strains would possibly make more comprehensive knowledge. Nevertheless, the slight differences did not affect the final conclusion. Taken together, our studies indicated that MoPEx5 and MoPEx7 mediate PTS1 and PTS2 peroxisomal pathways respectively and independently. Both PTS1 and PTS2 pathways played multiple roles in fungal development and pathogenicity, with the PTS1 pathway playing a more predominant role than the PTS2 pathway.
Supporting Information

**Figure S1** Vegetative growth of Δmopex5, Δmopex7 and wild type on medium with sodium acetate as sole carbon source.

**Figure S2** Tolerance to SDS of the Δmopex5, Δmopex7- and the wild type. The 5 mm mycelia discs were cultured on CM, CM supplied with 0.01% SDS or 0.02% SDS for 8 d, then the colonial diameters were measured and the relative inhibition rates were calculated. (A) The colonies of the strain cultured for 8 d. (B) the relative inhibition rate of Δmopex5 was higher significantly than those of the wild type and Δmopex7. The relative inhibition rate (%) = (the colonial diameter (DIC) on CM – DIC on (CM+SDS))/DIC on CM – 5).

**Figure S3** Tolerance to H2O2 of Δmopex5, Δmopex7 and the wild type during vegetative growth. The 5 mm mycelia discs were cultured on CM, CM supplied with 2.5 mM or 5 mM H2O2 for 10 d, then the colonial diameters were measured and the relative inhibition rates were calculated. (A) The colonies of the strain cultured for 10 d. (B) the relative inhibition rates of Δmopex5 and Δmopex7 were higher significantly that of the wild type. The relative inhibition rate (%) = (the colonial diameter (DIC) on CM – DIC on (CM+H2O2))/DIC on CM – 5).

**Figure S4** Relative expression of MoPEX5 and MoPEX7 during appressoria development. The conidia were allowed to form appressoria on a hydrophobic surface and sampled at different culture time points. The relative expressions of MoPEX5 and MoPEX7 in each sample were analyzed by using quantitative-PCR.

**Table S1** Predicted PTSs in enzymes involved in lipids degradation of *M. oryzae*. The open reading frames (ORF) encoding key enzymes involved in lipids degradation were first identified by searching the enzyme names against the *M. oryzae* genome database (http://www.broadinstitute.org/annotation/genome/magnaporthe_comparative/MultiHome.html), and then used as core sequences to re-search against the database using blastP procedure to pick out the ORFs predicted to encode the enzymes. The PTS1 motifs were predicted by the PTS1 predictor (http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp) with the parameter FUNGI-specific function [66,67]. The PTS2 motifs were predicted by searching the consensus (R/K)-(L/V/I)-X5-(H/Q)-(L/A) [13].

---

**Figure 12.** Expression of *MoPEX5* and *MoPEX7* during conidial germination and appressorium development. Promoter regions of *MoPEX5*, *MoPEX7* and were respectively fused to the GFP green fluorescent protein-encoding allele and introduced into *M. oryzae* strain Guy 11. Transformants carrying single-copy integrations were identified. Fluorescence of vegetative hyphae and during the appressorium development of germinating conidia on a hydrophobic surface of the transformants were analyzed and compared by epifluorescence microscopy. Control experiment in which a transformant expressing GFP under control of MPG1 [34] gene was allowed to undergo appressorium formation for 24 h. doi:10.1371/journal.pone.0055554.g012
Acknowledgments

We are grateful to Dr. Ping Wang (LSU Health Sciences Center, US) and Dr. Bo Dong (Zhejiang Agricultural Academy of Sciences, China) for language polishing.

References

1. Lazarow PB, Fujiki Y (1985) Biogenesis of peroxisomes. Annu Rev Cell Biol 1: 499–530.
2. Elgersma Y, Tabak HF (1996) Proteins involved in peroxisome biogenesis and functioning. Biochim Biophys Acta 1280: 209–203.
3. Vandenbosch H, Schegens HR, Wanders RJ, Tager JM (1992) Biochemistry of Peroxisomes. Annual Review of Biochemistry 61: 157–197.
4. Müller WH, Veldkamp TP, Kromer AJ, Westen HAB, Veldvoort LHM, et al. (1991) Localization of the Pathway of the Penicillin Biosynthesis in Penicillium chrysogenum. Embol Journal 10: 489–495.
5. Meier WH, Goffa J, Fekken S, Kiel JA, van den Berg MA, et al. (2010) Peroxisomes are required for efficient penicillin biosynthesis in Penicillium chrysogenum. Appl Environ Microbiol 76: 5702–5709.
6. Moser HW (2008) Molecular genetics of peroxisomal disorders. Front Biosci 5: D296–306.
7. Purdie PE, Lazarow PB (2001) Peroxisome biogenesis. Annual Review of Cell and Developmental Biology 17: 701–752.
8. Rucktaschel R, Girzalsky W, Erdmann R (2011) Protein import machineries of microbodies of the methylotrophic yeast Hansenula polymorpha. Fungal Genet Biol 48: 1405–1415.
9. van der Klei JJ, Hilbrands RE, Swaagting GJ, Waterham HR, Vrielin CG, et al. (1995) The Hansenula polymorpha PER3 gene is essential for the import of PTS1 proteins into the peroxisomal matrix. J Biol Chem 270: 17229–17236.
10. Saini RK, Todorov VL, Veenhuis M, Rachubinski RA (1995) Pex3p of the yeast Yarrowia lipolytica is an intraperoxosomal component of the matrix protein translocation machinery. J Cell Biol 131: 1435–1469.
11. Koch J, Franke J, Huber A, Ellinger A, Hartig A, et al. (2010) PEX11 family members are membrane elongation factors that coordinate peroxisomal proliferation and maintenance. J Cell Sci 123: 3389–3400.
12. Subramani S (1993) Protein Import into Peroxisomes and Biogenesis of the Peroxisome. Annual Review of Biochemistry 61: 157–197.
13. Hettema EH, Distel B, Tabak HF (1999) Import of proteins into peroxisomes. Biochim Biophys Acta 1451: 17–34.
14. Einwachter H, Sowinski S, Kunau WH, Schlielo W (2001) Yarrowia lipolytica Pex20p, Saccharomyces cerevisiae Pex1p/Pex21p and mammalian Pex3p, fulfill a common function in the early steps of the peroxisomal PTS2 import pathway. EMBO Rep 2: 1035–1039.
15. Otera H, Okamoto K, Tetsui K, Ikoma Y, Matsuda E, et al. (1998) Peroxisome targeting signal type 1 (PTS1) receptor is involved in import of both PTS1 and PTS2. studies with PEX5-defective CHO cell mutants. Mol Cell Biol 18: 388–399.
16. Hayashi M, Yagi M, Nito K, Kamada T, Nishimura M (2005) Differential contribution of two peroxisomal protein receptors to the maintenance of peroxisomal functions in Arabidopsis. J Biol Chem 280: 14829–14835.
17. Woodward AW, Bartel B (2005) The Arabidopsis peroxisomal targeting signal type 2 receptor PEX7 is necessary for peroxisome function and dependent on PEX5. Mol Biol Cell 16: 573–583.
18. Braverman N, Doh G, Gould SJ, Valle DH (1998) An isoform of pex5p, the human PTS1 receptor, is required for the import of PTS2 proteins into peroxisomes. Hum Mol Genet 7: 1195–1205.
19. Matsumura T, Otera H, Fujiki Y (2000) Disruption of the interaction of the terminal consensus Ser-Lys-Leu-related tripeptide of peroxisomal proteins requires carnitine-dependent transport of peroxisomal acetyl-CoA. Mol Microbiol 36: 1489–1495.
20. Kiel JA, van den Berg M, Bovenberg RA, van der Klei JJ, Veenhuis M (2004) Penicillium chrysogenum Pex3p mediates differential sorting of PTS1 proteins to microbodies of the methylotrophic yeast Hansenula polymorpha. Fungal Genet Biol 41: 708–720.
21. Watanabe H, Schegens HR, Wanders RJ, Tager JM, Lazarow PB (1992) Biochemistry of Peroxisomes. Annual Review of Biochemistry 61: 157–197.
22. Asakura M, Okuno T, Takano Y (2006) Multiple contributions of peroxisomal lipid metabolism in Magnaporthe grisea reveals a requirement for peroxisomal fatty acid beta-oxidation during appressorium-mediated plant infection. Mol Plant Microbe Interact 20: 475–491.

Author Contributions

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

Goh J, Leon J, Kim KS, Park J, Park SY, et al. (2011) The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in Magnaporthe grisea. PLoS One 6: e28220.

Wang JY, Tian YG, Zhang Z, Du X, Chai RY, et al. (2008) Fluorescent co-localization of PTS1 and PTS2 and its application in analysis of the gene function and the peroxisomal dynamic in Magnaporthe grisea. J Zhejiang Univ S 9: 802–810.

Goh J, Leon J, Kim KS, Park J, Park SY, et al. (2011) The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in Magnaporthe grisea. PLoS One 6: e28220.
50. Marzioch M, Erdmann R, Veenhuis M, Kunau WH (1994) PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. EMBO J 13: 4908–4918.

51. Zhang JW, Lazarow PB (1995) PEB1 (PAS7) in Saccharomyces cerevisiae encodes a hydrophilic, intra-peroxisomal protein that is a member of the WD repeat family and is essential for the import of thiolase into peroxisomes. J Cell Biol 129: 65–80.

52. Purdue PE, Yang X, Lazarow PB (1996) Pex18p and Pex21p, a novel pair of related peroxins essential for peroxisomal targeting by the PTS2 pathway. J Cell Biol 131: 1859–1869.

53. Otera H, Setoguchi K, Hamasaki M, Kumashiro T, Shimizu N, et al. (2002) Peroxosomal targeting signal receptor Pex5p interacts with cargoes and import machinery components in a spatiotemporally differentiated manner: conserved Pex5p WXXXF/Y motifs are critical for matrix protein import. Mol Cell Biol 22: 1639–1655.

54. Lee JR, Jang HH, Park JH, Jung JH, Lee SS, et al. (2006) Cloning of two splice variants of the rice PTS1 receptor, OsPex5pL and OsPex5pS, and their functional characterization using pex5-deficient yeast and Arabidopsis. Plant J 47: 457–466.

55. Brown LA, Baker A (2008) Shuttles and cycles: transport of proteins into the peroxisome matrix (review). Mol Membr Biol 25: 363–373.

56. Dodt G, Warren D, Becker E, Rehling P, Gould SJ (2001) Domain mapping of human PEX5 reveals functional and structural similarities to Saccharomyces cerevisiae Pex18p and Pex21p. J Biol Chem 276: 41769–41781.

57. Deng YZ, Ramos-Pamplona M, Naspi NI (2009) Autophagy-assisted glycolysis catabolism regulates asexual differentiation in Magnaporthe oryzae. Autophagy 5: 33–43.

58. Wang ZY, Thornton CR, Kershaw MJ, Li DR, Talbot NJ (2003) The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus Magnaporthe grisea. Molecular Microbiology 47: 1601–1612.

59. Tsai HF, Wheeler MH, Chang YC, Kwon-Chung KJ (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in Aspergillus fumigatus. J Bacteriol 181: 6469–6477.

60. Gurvitz A, Rottensteiner H, Kälppeläinen SH, Hartig A, Hiltunen JK, et al. (1997) The Saccharomyces cerevisiae peroxisomal 2,4-dienoyl-CoA reductase is encoded by the oleate-inducible gene SPS19. J Biol Chem 272: 22140–22147.

61. Coe JG, Murray LE, Dawes IW (1994) Identification of a sporation-specific promoter regulating divergent transcription of two novel sporation genes in Saccharomyces cerevisiae. Mol Gen Genet 244: 661–672.

62. Jones JM, Morrell JC, Gould SJ (2004) PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. J Biol Chem 265: 57–67.

63. Soundararajan S, Jedl G, Li X, Ramos-Pamplona M, Chua NH, et al. (2004) Woronin body function in Magnaporthe grisea is essential for efficient pathogenesis and for survival during nitrogen starvation stress. Plant Cell 16: 1546–1574.

64. Asiegbu FO, Cho S, Jeong JS, Dean RA (2004) Cloning, sequencing and functional analysis of Magnaporthe grisea MVP1 gene, a hex-1 homolog encoding a putative ‘woronin body’ protein. FEMS Microbiol Lett 230: 85–90.

65. Woloshuk CP, Soder HD, Vigil EL. (1983) Action of the antipenetrant, tricyclazole, on appressoria of Pyricularia oryzae. Physiological Plant Pathology 22: 245–247.

66. Neuberger G, Maurer-Stroh S, Eisenhaber B, Hartig A, Eisenhaber F (2003) Prediction of peroxisomal targeting signal 1 containing proteins from amino acid sequence. J Mol Biol 326: 501–592.

67. Neuberger G, Maurer-Stroh S, Eisenhaber B, Hartig A, Eisenhaber F (2003) Motif refinement of the peroxisomal targeting signal 1 and evaluation of taxon-specific differences. J Mol Biol 326: 567–579.