Pex14/17, a filamentous fungus-specific peroxin, is required for the import of peroxisomal matrix proteins and full virulence of *Magnaporthe oryzae*

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SUMMARY

Peroxisomes are ubiquitous organelles in eukaryotic cells that fulfil a variety of biochemical functions. The biogenesis of peroxisomes requires a variety of proteins, named peroxins, which are encoded by *PEX* genes. Pex14/17 is a putative recently identified peroxin, specifically present in filamentous fungal species. Its function in peroxisomal biogenesis is still obscure and its roles in fungal pathogenicity have not yet been documented. Here, we demonstrate the contributions of Pex14/17 in the rice blast fungus *Magnaporthe oryzae* (Mopex14/17) to peroxisomal biogenesis and fungal pathogenicity by targeting gene replacement strategies. Mopex14/17 has properties of both Pex14 and Pex17 with regard to its protein sequence. Mopex14/17 is distributed at the peroxisomal membrane and is essential for efficient peroxisomal targeting of proteins containing peroxisomal targeting signal 1. *MoPEX19* deletion leads to the cytoplasmic distribution of Mopex14/17, indicating that the peroxisomal import of Pex14/17 is dependent on Pex19. The knockout mutants of *MoPEX14/17* show reduced fatty acid utilization, reactive oxygen species (ROS) degradation and cell wall integrity. Moreover, Δ *mopex14/17* mutants show delayed conidial generation and appressorial formation, and a reduction in appressorial turgor accumulation and penetration ability in host plants. These defects result in a significant reduction in the virulence of the mutant. These data indicate that *MoPEX14/17* plays a crucial role in peroxisome biogenesis and contributes to fungal development and pathogenicity.

Keywords: fungal pathogenicity, *Magnaporthe oryzae*, *MoPEX14/17*, peroxisome.

INTRODUCTION

Rice blast disease, caused by the filamentous ascomycete *Magnaporthe oryzae*, is one of the most devastating diseases affecting global rice production (Dean *et al.*, 2005; Talbot, 2003; Wilson and Talbot, 2009). *Magnaporthe oryzae* is a model organism for the investigation of plant diseases caused by fungi, largely because of its economic importance, but also as a result of the experimental tractability of the fungus (Ebbole, 2007). The rice blast fungus attacks rice plants at all stages of development, such as leaves, stems, roots, nodes and panicles. When a conidium of *M. oryzae* attaches to a leaf under humid conditions, the conidium germinates rapidly and forms a specialized infection structure, known as an appressorium (Wilson and Talbot, 2009). Melanization of the appressorial cell wall and the accumulation of compatible solutes, such as glycerol, allow the generation of a high level of internal turgor pressure that is sufficient to rupture the plant cuticle (Howard *et al.*, 1991; de Jong *et al.*, 1997). After entering the plant, the fungus initially produces a thick and bulbous primary infection hypha (Heath *et al.*, 1990), which grows biotrophically and moves from cell to cell via plasmodesmata (Kankanala *et al.*, 2007); the secondary invasive hypha differentiates and colonizes rapidly by necrotrophic means inside the host tissue. After 3–5 days, visible lesions develop, from which the fungus will later release conidia for the next cycle (Talbot, 2003).

Peroxisomes are single membrane-bound organelles ubiquitously present in eukaryotic cells. They are involved in various important metabolic processes and are highly dynamic with strong plasticity (Islinger *et al.*, 2010). They play a major role in lipid metabolism and the production and disposal of reactive oxygen species (ROS), and characteristically contain the enzymes for fatty acid β -oxidation and for the formation of hydrogen peroxide-producing oxidases and catalase. The other functions of peroxisomes vary according to cell type and cell-specific metabolic demands. In humans, the important reactions occurring in peroxisomes include fatty acid α -oxidation, the degradation of D-amino acids, the catabolism of purines and the biosynthesis of ether lipids, polyunsaturated fatty acids and bile acids (Wanders and Waterham, 2006). Defects in peroxisomes lead to multiple

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developmental diseases, the most severe of which is Zellweger syndrome, which causes death in early childhood (Faust *et al.*, 2005; Wanders and Waterham, 2005). In plants, loss of peroxisomal function causes embryo lethality, suggesting an essential role in growth and development (Fan *et al.*, 2005; Tzafrir *et al.*, 2004). Peroxisomes also play vital roles in filamentous fungi, as a variety of fungal metabolites are at least partially synthesized inside peroxisomes. The peroxisome-deficient fungi fail to grow in minimal medium containing fatty acids as sole carbon source and exhibit delayed growth and aberrant organelle morphologies, suggesting fundamental roles of peroxisomes in the growth of filamentous fungi (Bonnet *et al.*, 2006; Hynes *et al.*, 2008; Idnurm *et al.*, 2007; Stehlik *et al.*, 2014). Peroxisomes are also involved in secondary metabolism, including the biosynthesis of penicillin, AK toxin and paxilline, and sexual development (Asakura *et al.*, 2006; Bartoszewski *et al.*, 2011; Bonnet *et al.*, 2006; Imazaki *et al.*, 2010; Kimura *et al.*, 2001; Peraza-Reyes *et al.*, 2008; Saikia and Scott, 2009).

Most of the genes involved in peroxisome biogenesis, so-called *PEX* genes, encode proteins named peroxins (Distel *et al.*, 1996). During recent decades, more than 30 peroxins have been identified that are required for either peroxisome assembly or proliferation (Distel *et al.*, 1996; Nuttall *et al.*, 2011; Pieuchot and Jedd, 2012). Peroxisomal matrix proteins are targeted to peroxisomes by one of two well-characterized peroxisomal targeting signals, PTS1 or PTS2, and are imported in a folded state or as oligomers (Brocard and Hartig, 2006; Glover *et al.*, 1994; Gould *et al.*, 1989; Lazarow, 2006; McNew and Goodman, 1994; Swinkels *et al.*, 1991; Walton *et al.*, 1995). Pex5 and Pex7 are specific receptors that recognize PTS1 and PTS2, respectively (Braverman *et al.*, 1997; Brocard *et al.*, 1994; Dodt and Gould, 1996; Lazarow, 2006; Van der Leij *et al.*, 1993). Both PTS receptors are predominantly soluble proteins that bind the cargos in cytoplasm and target them to the peroxisomal membrane, where the matrix proteins are imported into the organelle. The docking of both cargo-loaded receptors onto the peroxisomal membrane requires three membrane-associated peroxins: Pex13, Pex14 and Pex17. These three peroxins are thought to form a receptor-docking complex responsible for the initial binding of the receptor–cargo complex at the peroxisomal membrane (Lazarow, 2003). Pex13 and Pex14 bind the receptors, whereas Pex14 also interacts with Pex17 (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Elgersma *et al.*, 1996; Gould *et al.*, 1996). The docking complex might also cooperate with, or be part of, the translocation machinery for matrix proteins.

As the third component of the docking complex, Pex17 is present in yeast species, but has not been detected so far in higher eukaryotes, including humans, plants and filamentous fungi (Rucktäschel *et al.*, 2010). More interestingly, the filamentous fungi have been found recently to contain a unique peroxin,

Pex14/17 (or Pex33), which seems to be a fusion protein between Pex14 and Pex17, and may be functionally equivalent to the yeast Pex17 (Kiel *et al.*, 2006; Managadze *et al.*, 2007; Opaliński *et al.*, 2010). In *Penicillium chrysogenum*, Pex14/17 possesses N-terminal and C-terminal domains homologous to those of Pex14 and Pex17, respectively, is required for efficient PTS1 and PTS2 import, and is necessary for penicillin production and the production of asexual spores (Opaliński *et al.*, 2010). The *Neurospora crassa* orthologue of Pex14/17 is essential for the biogenesis of glyoxysomes and Woronin bodies. In *Podospira anserina*, Pex14/17 plays different roles during meiosis and sporulation (Peraza-Reyes *et al.*, 2011).

In recent years, peroxisomal metabolism has been demonstrated to be crucial for pathogenicity in several plant-pathogenic fungal species. Thus, the deletion of *PEX6* damages peroxisomal metabolism and impairs fungal infection in *Colletotrichum lagenarium* and *M. oryzae* (Asakura *et al.*, 2006; Kimura *et al.*, 2001; Wang *et al.*, 2007; Wilson and Talbot, 2009). *PEX5* and *PEX7* are both required for development and pathogenicity in *M. oryzae*, meanwhile, *PEX5* influences the development and pathogenicity more than *PEX7*. (Goh *et al.*, 2011; Wang *et al.*, 2013). *PEX5* and *PEX6*, rather than *PEX7*, are crucial to survival and virulence in *Fusarium graminearum* (Min *et al.*, 2012). *PEX13* has been found to be indispensable for infection in *Colletotrichum orbiculare* (Fujihara *et al.*, 2010). As a fungal-specific peroxin, however, Pex14/17 has not been investigated in any fungal pathogens; hence, its function in peroxisomal biogenesis and whether it plays a role in pathogenicity are still unknown. To thoroughly evaluate the role of peroxisomes in fungal pathogenesis, in the present work, we functionally characterize *PEX14/17* in the rice blast fungus *M. oryzae* using gene replacement strategies.

EXPERIMENTAL PROCEDURES

Strains, cultivation and transformation

Magnaporthe oryzae wild-type Guy-11 (Nottingham and Silue, 1992) and all transformants were routinely cultured on complete medium (CM) at 28°C for 3–14 days (Crawford *et al.*, 1986; Talbot *et al.*, 1993). Lipid medium, glucose medium and sodium acetate medium were prepared as described previously (Wang *et al.*, 2013). All fungal transformants were generated by *Agrobacterium tumefaciens*-mediated transformation (A₁MT), as described previously (Rho *et al.*, 2001). CM plates containing 250 µg/mL hygromycin B (Roche, Mannheim, Germany), 200 µg/mL glufosinate-ammonium (Sigma, St Louis, MO, USA) or 800 µg/mL G418 (Sigma) (Li *et al.*, 2014) were used to screen the corresponding transformants. Cell wall integrity was tested by growing the strains on CM supplemented with 100 µg/mL Congo red or 50 µg/mL calcofluor white. The tolerance of the strains to ROS was evaluated by growth on CM containing 2.5 mM H₂O₂ or 1 mM methyl viologen. For dry weight measurement, mycelia of the strains were collected by culture in liquid CM with shaking

at 150 rpm at 28 °C for 4 days, followed by drying at 37 °C. The production of melanin was measured by the optical density at 415 nm (OD₄₁₅).

Sequence analysis

The Pex14/17 homologues were identified by searching the Genbank and fungal genome databases (<http://www.broadinstitute.org/scientific-community/data>). The coding sequences of *MoPEX14/17* were determined by amplification of the cDNA fragment (with the primer set 14/17CDS1 and 14/17CDS4) from total RNA and sequencing. Sequence alignments were performed using the CLUSTALW method, and imported into the software GeneDoc 2.0 for type setting and into MEGA version 6.0 to establish the phylogenetic trees.

Nucleic acid manipulation and construction of fluorescent fusions

Genomic DNA was isolated using the cetyltrimethylammonium bromide method (Talbot *et al.*, 1993). Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and employed to synthesize cDNA using AMV Reverse Transcriptase (Takara Bio, Otsu, Japan). Polymerase chain reaction (PCR), restriction digestion, gel electrophoresis and ligation reactions were performed using standard procedures. Transcript abundance was analysed by quantitative reverse transcription PCR on the 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA) with the β -tubulin gene (MGG_00604) for normalization. p1300BMGFPA [containing GFP-PTS1, a PTS1-tagged enhanced green fluorescent protein (eGFP)], p1300BMGFPB (containing GFP-PTS2, a PTS2-tagged eGFP) and p1300NMcherryA (a modified vector formed by substituting DsRED-PTS1 in p1300NMRFP with mCherry-PTS1) were used as peroxisomal markers (Li *et al.*, 2014; Wang *et al.*, 2008).

To track the cellular localization of Mopex14/17, the coding sequence of *MoPEX14/17* was amplified using the primer set 14/17CDS2 and 14/17CDS3 from the total RNA as template, and introduced into p1300BMGFP-C (Li *et al.*, 2014) by *EcoRI/SmaI* digestion to produce the fusion vector p1300BMGFP-PEX14/17, in which the GFP-Mopex14/17 fusion could be expressed under the control of the *MPG1* promoter. To mark the position of the peroxisomes, p1300NMcherryA was co-integrated into the p1300BMGFP-PEX14/17 transformants.

Gene deletion and mutant recovery

For gene deletion, at least a 1.5-kb upstream fragment (*EcoRI/KpnI*, with primer set Pko17upF and Pko17upR) and downstream fragment (*HindIII/XbaI*, with primer set Pko17dnF and Pko17dnR) for *MoPEX14/17* were amplified from genomic DNA and inserted into p1300-KO (Li *et al.*, 2014) to generate the gene replacement vector pKO-PEX14/17. The transformants resistant to hygromycin B were harvested and initially screened by genomic PCR with the primer sets 14/17CDS1 and 14/17CDS4, 14/17Y21 and SeqUP, 14/17Y22 and SeqDN, and HPHCK1 and HPHCK2. The potential deletants were further confirmed by quantitative reverse transcription PCR using the primer set 14/17RTF and 14/17RTR.

For mutant recovery, p1300BMGFP-PEX14/17 was introduced into a confirmed deletion mutant. The resulting transformants were picked up by glufosinate-ammonium resistance and checked by genomic PCR. The

candidate revertants were further confirmed by quantitative reverse transcription PCR, and one confirmed revertant was used in phenotypical analysis.

Pathogenicity tests, infectious structure observation and 3,3'-diaminobenzidine (DAB) staining

Two-week-old rice CO39 and 7-day-old barley ZJ-8 were used in pathogenicity tests. For spray inoculation, the conidia were harvested from 10-day-old CM cultures and resuspended at 1×10^5 conidia/mL with 0.25% (w/v) gelatin (Talbot *et al.*, 1993). For inoculation on detached leaves, 5-mm mycelial plugs were placed on leaf segments, and incubated at 28 °C in darkness for 24 h and, subsequently, in 24-h light for 3 days. To observe infectious structures, the inoculated barley leaves were discoloured with lactic acid, heated at 65 °C for 2 h and examined microscopically. To detect ROS accumulation in infected host cells, the DAB staining approach was used. Barley leaves inoculated with a conidial suspension at a concentration of 2×10^5 conidia/mL were detached and incubated in 1 mg/mL DAB (Sigma-Aldrich, St Louis, MO, USA) solution in darkness at 28 °C for 8 h, decolorized in boiling ethanol (96%) for 20 min and observed under a light microscope.

Measurement of conidial germination, appressorial formation and turgor generation

Conidia harvested from 10-day-old CM cultures were resuspended at 1×10^5 /mL. Aliquots (20 μ L) of conidial suspensions were incubated on a plastic coverslip at 28 °C for 24 h to allow germination and appressorial formation. Appressoria formed at 24 h post-incubation were used in an incipient cytorrhysis assay to measure appressorial turgor. The experiments were replicated three times and more than 200 appressoria were counted for each strain.

Fluorescence microscopy

The fluorescence of GFP and mCherry was detected using a Leica SP2 Confocal System (Mannheim, Germany), with excitation at 488 nm and emission at 520 nm for GFP, and excitation at 558 nm and emission at 583 nm for mCherry.

RESULTS

Identification of *MoPEX14/17* gene in *M. oryzae*

The analysis of the genomic data revealed that *M. oryzae* lacks Pex17, but contains Pex14/17 (MGG_01081, assigned as Mopex14/17 in the present work), which exhibits similarity to both Pex14 and Pex17. Comparison of Mopex14/17 with the orthologue from fungi and other organisms indicated that this protein is specific for filamentous fungi. cDNA sequencing confirmed that the open reading frame (ORF) of *MoPEX14/17* is 1101 bp in length, with one intron and two exons, and encodes a polypeptide of 367 amino acid residues. The predicted protein sequence of Mopex14/17 shows 40% amino acid identity with the hypothetical Pex14/17 from *Colletotrichum graminicola* (XP_008096954.1) and 38% amino acid identity with that from *Fusarium oxysporum* (ENH74148.1) (Fig. 1A,B). The N-terminus of Mopex14/17 is similar to the highly conserved region present in the N-terminus of

A **B** **C** **D** **E**

Fig. 1 Sequence analysis of Pex14/17 homologues. (A) Multiple sequence alignment of Mopex14/17 with homologous proteins from other fungal species. The identical amino acids are highlighted against a black background, the conserved residues on a dark grey background and the similar residues on a light grey background. The domain exhibiting homology to the N-terminal domain of Pex14 is denoted by a red line. Other identified domains are denoted by lines above the sequences [purple, PEST sequence; orange, coiled-coil domain; blue, hydrophobic domain, possible transmembrane (TM) domain]. (B) Phylogenetic relationship of Pex14/17 homologues calculated using the neighbor-joining method with the MEGA 6.0 program according to the alignment. (C) Sequence comparison of C-terminal hydrophobic regions (CTHRs) of Mopex14/17 and Scpex17. This motif is absent in Pex14 proteins. (D) Sequence comparison of the conserved N-terminal regions of Mopex14/17, Mopex14 and ScPex14. (E) Schematic representation of Mopex14/17, Mopex14 and Scpex17. The Pex5-binding site in Mopex14 and the corresponding homologous region in Mopex14/17 are shown in red. A hydrophobic region present in all three proteins is shown in blue. Predicted coiled-coil domains are shown in orange, and putative PEST regions, involved in the regulation of protein levels, are shown in brown. The CTHR conserved in Mopex14/17 and Scpex17 is indicated in green. Abbreviations and accession numbers of the sequences are as follows: Anpex14/17 (XP_663862.1) from *Aspergillus nidulans*; AoPex14/17 (XP_001821808.1) from *Aspergillus oryzae*; BcPex14/17 (XP_001557279.1) from *Botrytis cinerea*; CgPex14/17 (EFQ32934.1) from *Colletotrichum graminicola*; FgPex14/17 (XP_011321285.1) from *Fusarium graminearum*; Fopex14/17 (ENH74148.1) from *Fusarium oxysporum*; GgPex14/17 (XP_009220404.1) from *Gaeumannomyces graminis*; Mopex14/17 (XP_003717857.1) from *M. oryzae*; NcPex14/17 (XP_956984.3) from *Neurospora crassa*; PaPex14/17 (CDP27033.1) from *Podospora anserina*; Prpex14/17 (CDM35356.1) from *Penicillium roqueforti*; ScPex14/17 (CAA96116) from *Saccharomyces cerevisiae*; Vdpex14/17 (XP_009648065.1) from *Verticillium dahliae*.

Pex14, which is responsible for Pex5p binding, whereas its C-terminus shows weak similarity with that in Pex17 (Fig. 1C,D). Although the homology of Mopex14/17 to Pex14 is only evident in the short N-terminal domain, both proteins harbour two coiled-coil domains (Fig. 1E) and thus share a more extended structural similarity. Based on these similarities, Pex14/17p might either constitute an ancillary, redundant isoform of Pex14, or act independently of Pex14 in peroxisome biogenesis. However, Mopex14/17 has a C-terminal homology region (CTHR), which is exclusively present in fungal Pex14/17 and yeast Pex17, but absent in Pex14, suggesting another possibility that Mopex14/17 functions as Pex17 (Fig. 1A,E).

MoPEX14/17 is up-regulated by lipids and appressorial differentiation

In order to investigate the temporal and spatial expression of *MoPEX14/17* during fungal development, we assessed the relative transcription of *MoPEX14/17* in the wild-type strain by quantitative reverse transcription PCR. The results revealed that *MoPEX14/17* could be up-regulated by Tween 80, olive oil and sodium acetate (Fig. 2A), suggesting that *MoPEX14/17* participates in fat metabolism. During conidial germination, germ tube elongation and initial emergence of appressoria, the expression of *MoPEX14/17* increased gradually, with a peak at the initial emergence of appressoria at 6 h, and then decreased gradually (Fig. 2B).

Fig. 2 Expression dynamics of *MoPEX14/17*. (A) Relative transcription levels of *MoPEX14/17* on minimal medium (MM) with glucose, Tween 80, olive oil or sodium acetate as sole carbon source. Double asterisks indicate significant differences from the transcription level on MM ($P < 0.01$). (B) Relative transcription levels of *MoPEX14/17* during conidial germination and appressorial formation. Total RNAs were extracted from *Magnaporthe oryzae* mycelium grown in different media or conidia incubated on plastic membrane. Transcript abundance was measured by quantitative reverse transcription polymerase chain reaction (PCR) and normalized to the β -tubulin gene.

MoPEX14/17 is distributed at peroxisomes via MoPEX19

To visualize the subcellular localization of Mopex14/17, the strain expressing an eGFP-tagged version of the protein was constructed and examined by fluorescence microscopy. The GFP-Mopex14/17 transformants emitted fluorescence in a punctate pattern, which was congruent in most instances to the red fluorescence of mCherry-PTS1, a peroxisomal marker, suggesting the peroxisomal localization of Mopex14/17. In amplified images, GFP-Mopex14/17 appeared as green circles which surrounded the red points of mCherry-PTS1 which were distributed inside peroxisomes, indicating that Mopex14/17 is a component of the peroxisome and localized in the peroxisomal membrane (Fig. 3A).

Pex19p is a receptor and chaperone for peroxisomal membrane proteins (PMPs), and is indispensable for the assembly of multiple PMPs on the peroxisomal membrane, and in *M. oryzae* Pmp47 (Li *et al.*, 2014). To investigate whether Mopex14/17 is imported via Mopex19, we observed the distribution of Mopex14/17 on deletion of *MoPEX19*. In $\Delta mopex19$ mutants, a clear import defect of Mopex14/17 was found, in which the eGFP-tagged Mopex14/17 showed a cytoplasmic distribution. These data indicate that the import of Mopex14/17 requires *MoPEX19* (Fig. 3B).

Targeted disruption of MoPEX14/17

To determine the functions of *MoPEX14/17*, we performed gene deletion. The targeted gene deletion vector PKO-PEX14/17 was introduced into the wild-type strain Guy-11 (Fig. 4A), and the resulting hygromycin-resistant strains were tested by genomic PCR and quantitative reverse transcription PCR. As expected, the fragment within the *MoPEX14/17* encoding sequence could be detected from the wild-type, but not $\Delta mopex14/17$ mutants (Fig. 4B). For mutant complementation, a complement vector p1300BMGFP-PEX14/17, which contains an eGFP-tagged full-length coding fragment of *MoPEX14/17*, was integrated into the $\Delta mopex14/17$ mutant; the positive complement transformants were confirmed by genomic PCR and reverse transcription PCR (Fig. 4C). Two confirmed $\Delta mopex14/17$ mutants ($\Delta mopex14/17-4$ and $\Delta mopex14/17-54$) and one confirmed complemented transformant (*mopex14/17-com*) were used for subsequent phenotypic analysis.

Deletion of MoPEX14/17 suppresses conidial production

Cultured on CM, the $\Delta mopex14/17$ mutants showed no significant difference from the wild-type and complemented strains with regard to colony morphology, pigmentation and aerial hyphal development (Fig. 5A,B), and only a slight reduction in radial growth and dry weight (Fig. 5C,D). The melanin production of $\Delta mopex14/17$ mutants was also unaltered from that of the wild-

type when cultured in liquid CM (Fig. 5E,F). This is in contrast with $\Delta mopex19$, which formed typical small and bald colonies on CM (Li *et al.*, 2014). However, the conidiation of the $\Delta mopex14/17$ mutants was reduced to 40% of that of the wild-type and complemented strains cultured on CM for 6 days (Fig. 5G,H). These results indicate that *MoPEX14/17* is involved in the conidial production of *M. oryzae*.

MoPEX14/17 is required for efficient import of matrix protein into the peroxisome

To test the role of *MoPEX14/17* in peroxisome biogenesis, we analysed the localization of two eGFP-tagged peroxisome marker proteins: GFP-PTS1 and GFP-PTS2 (Li *et al.*, 2014; Wang *et al.*, 2008). The two marker proteins were found to be well distributed in peroxisomes in the wild-type strain. Deletion of *MoPEX14/17* inhibited the PTS1 import pathway and resulted in bimodal localization of GFP-PTS1 between the cytosol and peroxisomes (Fig. 6A), although this situation differed from that found in other *M. oryzae* pex mutants, such as $\Delta mopex5$, where PTS1-containing proteins were completely mislocalized to the cytosol (Li *et al.*, 2014; Wang *et al.*, 2007). However, the distribution of GFP-PTS2 exhibited no obvious difference in $\Delta mopex14/17$ mutants compared with the wild-type strain (Fig. 6B), unlike that in $\Delta mopex7$ (Li *et al.*, 2014; Wang *et al.*, 2007). In addition, the number, sizes and distribution profile of peroxisomes indicated by GFP-PTS1 or GFP-PTS2 were unchanged in the mutants compared with the wild-type. These observations indicate that *MoPEX14/17* is required for the efficient import of PTS1-containing proteins, but not for the existence of the organelles.

MoPEX14/17 is necessary for pathogenicity in *M. oryzae*

To test the contribution of *MoPEX14/17* to blast disease, spores of the $\Delta mopex14/17$ mutant and wild-type Guy-11 were harvested, adjusted to uniform concentrations and inoculated onto 14-day-old seedlings of the blast-susceptible rice cultivar CO39 and 7-day-old barley cultivar ZJ-8 (Fig. 7). The symptoms caused by $\Delta mopex14/17$ were strongly reduced on both barley and rice leaves, compared with those induced by the wild-type, and also contrasted with those of $\Delta mopex19$, which was completely non-pathogenic (Fig. 7A–C). Using image analysis software (ImageJ), we quantified and statistically analysed the lesion density and sizes, and found that the lesion numbers and sizes generated by $\Delta mopex14/17$ were significantly lower than those generated by the wild-type (*t*-test, $n > 100$, $P < 0.01$, Fig. 7D). Similar results were also obtained from the inoculation with mycelial plugs on barley leaves. Meanwhile, the complemented strain *mopex14/17-com*, which possessed an eGFP-tagged *MoPEX14/17*, was able to restore the full virulence of the mutants. These results indicate

Fig. 3 Cellular localization of Mopex14/17. (A) Fluorescence microscopy of co-transformants of GFP-Mopex14/17 and mCherry-PTS1 in *Magnaporthe oryzae* wild-type. The fluorescence of GFP-Mopex14/17 was localized in the membrane of peroxisomes, whereas the red fluorescence of mCherry-PTS1 was distributed in the matrix of peroxisomes. Arrows of the same colour indicate the same peroxisome at different magnifications. (B) GFP-Mopex14/17 exhibited a cytoplasmic distribution in $\Delta mopex19$ mutants. DIC, differential interference contrast. Bars, 2 μ m.

that the virulence of the fungus is reduced by deletion of *MoPEX14/17*.

To explore the reasons for the virulence reduction in $\Delta mopex14/17$, we observed the invasion structure by histological microscopy. Inoculated on host leaves, $\Delta mopex14/17$ mutants

were capable of forming appressoria in an identical manner, but with a significantly reduced frequency, to that of the wild-type strain (Fig. 7E). At 24 h post-inoculation (hpi), over 95% of the wild-type appressoria, but only 20% of the $\Delta mopex14/17$ appressoria, penetrated the host cells and formed visible primary

Fig. 4 *MoPEX14/17* gene deletion and mutant complementation. (A) Diagram showing that the 1.1-kb *MoPEX14/17* coding region was replaced by the 1.36-kb *HPH* cassette. Scale bar, 500 bp. The locations of the primers are indicated with arrows, and primers in the same color were used as a pair. P1, *HPHCK1*; P2, *HPHCK2*; P3, 14/17CDS1; P4, 14/17CDS4; P5, 14/17YZ1; P6, SeqUP; P7, SeqDN; P8, 14/17YZ2. (B) Genomic polymerase chain reaction (PCR) was used to validate the deletion of *MoPEX14/17*. The Upstream fragment, Dnstream fragment, *MoPEX14/17* and *HPH* were amplified with the primer pairs P5/P6, P7/P8, P3/P4 and P1/P2, respectively. (C) Confirmation of the mutants by transcriptional analysis. *MoPEX14/17* transcripts were detected in the wild-type (Guy-11) and complemented strain *mopex14/17-com* using quantitative reverse transcription PCR, but completely undetectable in the mutants Δ *mopex14/17-14* and Δ *mopex14/17-54*. Double asterisks indicate significant differences from the transcriptional level of the wild-type ($P < 0.01$).

Fig. 5 Vegetative growth and conidiation of Δ *mopex14/17* mutants. (A, B) The Δ *mopex14/17* mutants, wild-type, complemented strains and Δ *mopex19* were cultured on complete medium (CM) at 28 °C for 6 days on 6-cm culture plates. (C) Measurement and statistical comparison of radial growth of the strains. (D) The dry weights of mycelia from Guy-11, Δ *mopex14/17* mutants, *mopex14/17-com* and Δ *mopex19* were compared by growth in liquid CM with shaking at 150 rpm at 28 °C for 4 days. (E, F) The cultures of the strains were observed and the pigmentation of the cultures was quantitatively compared by measurement of the optical density at 415 nm (OD_{415}). (G) The conidial generation of the strains was examined by light microscopy. Bar, 50 μ m. (H) Statistical analysis of conidia produced on CM culture at 28 °C for 6 days. Means and standard errors were calculated from three independent replicates. Single asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences from the wild-type ($P < 0.01$).

Fig. 6 Distribution of the peroxisomal matrix proteins in $\Delta mopex14/17$ mutants. GFP-PTS1 and GFP-PTS2 were integrated into the wild-type, $\Delta mopex14/17$, $\Delta mopex5$ and $\Delta mopex7$. Conidia of the transformants were harvested from 5-day-old complete medium (CM) plates and detected using confocal fluorescence microscopy. GFP-PTS1 was predominantly present in a punctate pattern (peroxisomal distribution) in the wild-type, but was bimodally distributed in peroxisomes and cytoplasm in $\Delta mopex14/17$. As controls, GFP-PTS1 in $\Delta mopex5$ and GFP-PTS2 in $\Delta mopex7$ were completely cytoplasmically distributed. Bar, 5 μ m.

infection hyphae, indicating that the ability of the appressoria of $\Delta mopex14/17$ to penetrate the host cuticle was reduced compared with that of the wild-type. At 48 hpi, the $\Delta mopex14/17$ mutant had only just started to colonize neighbouring cells, whereas Guy-11 had become well established, with bulbous hyphae fully ramified in host tissues. From these observations, we conclude that the virulence phenotype of the $\Delta mopex14/17$ mutant is associated with a reduced ability of the $\Delta mopex14/17$ mutant to proliferate within host tissues, in addition to a reduced capacity to form mature appressoria and undergo successful penetration.

Deletion of *MoPEX14/17* retards conidial germination and appressorial formation, and reduces appressorial turgor generation

The conidia of the $\Delta mopex14/17$ mutant and the wild-type were incubated on a plastic membrane to allow appressoria formation. The rates of conidial germination and appressorial formation were both found to be reduced in $\Delta mopex14/17$ mutants at the initial stages, although the final percentages equalled those of the wild-type (Fig. 8A,B). Appressorium-mediated penetration requires very high internal turgor pressure. To determine whether the turgor pressure of $\Delta mopex14/17$ appressoria was altered compared with that of wild-type appressoria, we performed incipient cytorrhysis.

Treatment with 0.5, 1.0, 2.0 and 3.0 M glycerol led to appressorial collapse in the $\Delta mopex14/17$ mutants at significantly higher levels than that in the wild-type and complemented strains (Fig. 8C). These data indicate that *MoPEX14/17* plays a role in conidial germination, appressorial formation and appressoria turgor generation, all of which are key factors in host penetration.

MoPEX14/17 mutant is more sensitive to ROS and involved in lipid metabolism

Saccharomyces cerevisiae Pex17 is involved in the response to oxidative stress. To investigate whether *MoPEX14/17* exhibits the same function, the tolerance of $\Delta mopex14/17$ mutants to ROS was first compared with the wild-type and the complemented transformants *mopex14/17-com* on medium inducing oxidative stress. On exposure to 2.5 mM H_2O_2 or 1 mM methyl viologen, the radial growth of *MoPEX14/17* mutants was inhibited by 28.9% (H_2O_2) or 31.1% (methyl viologen), significantly greater than the values for the wild-type strain (24.0% by H_2O_2 and 28.0% by methyl viologen) (Fig. 9A,B). Meanwhile, the complemented strain *mopex14/17-com* was as resistant to oxidative stress as the wild-type strain, indicating the involvement of the *MoPEX14/17* gene in the tolerance to oxidative stress. The ROS burst in plant cells infected by pathogens is a major barrier for further invasion of pathogens, and thus reduced ROS tolerance may be one reason

Fig. 7 Pathogenicity test of the *MoPEX14/17* deletion mutants. (A) Spray inoculation with conidial suspension (1×10^5 conidia/mL) on 7-day-old barley cultivar ZJ-8. The symptoms were recorded at 3 days post-inoculation (dpi). (B) Detached barley leaves inoculated with 5-mm mycelial plugs were cultured for 4 days. (C) Spray inoculation with conidial suspension (1×10^5 conidia/mL) on 2-week-old rice cultivar CO39, recorded at 7 dpi. (D) The numbers of lesions on 5-cm-length leaves were counted and statistically analysed. Error bars represent the deviation from three replicates and double asterisks indicate significant differences at the $P < 0.01$ level. (E) Droplet-inoculated barley leaves were sampled at 18, 24 and 48 h post-inoculation, discoloured and examined under a light microscope. The conidia (C), appressoria (A) and invasive hyphae (IH) are marked. Bar, 20 μ m.

Fig. 8 Conidial germination and appressorial formation of $\Delta mopex14/17$. The conidial germination rates (A) and appressorial formation rates (B) of $\Delta mopex14/17$, wild-type and complemented strains on a hydrophobic surface were calculated. (C) Cytorrhysis assay to compare the appressorial turgor genesis in the 24-h appressoria of $\Delta mopex14/17$ and the controls. Means and standard errors were calculated from three independent replicates (at least 100 conidia for each). Single asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$.

Fig. 9 Defects in reactive oxygen species (ROS) elimination and lipid utilization in $\Delta mopex14/17$. (A) Mycelial growth of the *MoPEX14/17* mutants under oxidative stress. The wild-type, $\Delta mopex14/17$ and complemented strains were cultured on complete medium (CM) and CM supplemented with 2.5 mM H_2O_2 or 1 mM methyl viologen at 28 °C for 5 days. (B) The growth inhibition rates calculated based on the colony diameters were statistically analysed. Inhibition rate = (diameter of untreated strain – diameter of treated strain)/(diameter of untreated strain $\times$ 100%). (C) Detached barley leaves were inoculated with the tested strains, stained with 3,3'-diaminobenzidine (DAB) at 28 and 40 h post-inoculation (hpi) and observed under a light microscope. A, appressorium; IH, infectious hypha. Bar, 25 μ m. (D) The strains were cultured on minimal medium with 1% glucose, 0.5% (v/v) olive oil or 0.5% (v/v) Tween 80 as sole carbon source at 28 °C for 6 days. (E) The colony diameters were measured and statistically analysed. Error bars represent the deviation calculated from three replicates; single asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$.

Fig. 10 Tolerance test of *Δmopex14/17* to cell wall interference agents. (A) The wild-type, *Δmopex14/17* mutants and complemented strains were cultured on complete medium (CM) supplemented with 100 μg/mL Congo red or 50 μg/mL calcofluor white for 5 days. (B) The colony diameters of the tested strains were measured to calculate the inhibition rates and statistically analysed. Error bars represent the deviation from three replicates, and double asterisks indicate significant differences at the $P < 0.01$ level.

for the virulence defects of the mutants. To confirm this speculation, we compared the ROS burst in barley cells infected by *Δmopex14/17* mutants and the wild-type via the DAB staining approach (Fig. 9C). Most of the host cells infected by the mutants were stained brown by DAB, which initially presented around the infection hyphae at 28 hpi and filled the entire infected host cell at 40 hpi. However, the cells infected by the wild-type showed little DAB staining or light staining. Collectively, these results suggest that deletion of *MoPEX14/17* reduces the resistance of *M. oryzae* to ROS, both *in vitro* and within host cells, which is probably a reason for the defects in the development of the mutants inside the host tissue.

To assess the effects of *MoPEX14/17* deletion on peroxisomal lipid metabolism, we investigated the lipid utilization capacity of the mutants. On medium with Tween 80 or olive oil as sole carbon source, the development of *Δmopex14/17* mutants was strongly decreased compared with that of the wild-type and the complemented strain, indicating disruption of lipid metabolism in the mutants (Fig. 9D,E). The results suggest that deletion of *MoPEX14/17* affects lipid metabolism.

Deletion of *MoPEX14/17* alters the integrity of the cell wall

To confirm whether *MoPEX14/17* deletion alters the integrity of the cell walls, we observed the tolerance of the *Δmopex14/17* mutants to CR medium (Congo red was added to CM to 100 μg/mL) and CFW medium (calcofluor white was added to CM to 50 μg/mL). Whether on CR or CFW medium, the colony diameters of the mutants were smaller than those of the wild-type and complemented strains (Fig. 10). This indicates that *MoPEX14/17* plays a role in the integrity of the cell walls of *M. oryzae*.

DISCUSSION

As a peroxin specifically present in filamentous fungi, Pex14/17 has already been proven to be involved in the import of matrix proteins and penicillin biosynthesis in *P. chrysogenum*, and the biogenesis of peroxisomes and Woronin bodies in *N. crassa* (Managadze *et al.*, 2010; Opaliński *et al.*, 2010). In the present study, we functionally analysed Pex14/17 from *M. oryzae* using targeted gene deletion methods, and found that the loss of *MoPEX14/17* led to a plethora of developmental defects in conidium production, germination, appressorium formation, penetration

Table 1 Primers used in this study.

Name	Sequence (5'–3')
14/17CDS1	TCCCCAAAAGGACATCAGACACAA
14/17CDS4	CAACACGTGACAAGAACCCTCACT
14/17CDS2	GACGAGCTGTACAAGCCCGGTGTGGCCGACCC
	TGACAAGAAAGAG
14/17CDS3	CATGATTACGAATTCGAGCTCTCATCGCAACGT
	GGACCCCTGCGG
Pko17upF	CGGAAATCAAGGGATAAATGATAAAGATAGGC
Pko17upR	GGGGTACCGAATGAGGATTGTAAATGTTGCTG
Pko17dnF	GCCTAGATAATGAATGGACGAAATACAGCA
Pko17dnR	CCCAAGCTTGTGACTATACATGGGCATTGTA
14/17YZ1	GTGGGTGCGGCACGATCAACAAGA
14/17YZ2	AAGACGGCGATGAAGTGTTCAC
HPHCK1	TTCGCCCTTCTCCTTTATTCA
HPHCK2	GCTTCTGCGGGCGATTGTGTACG
SeqUP	ACTCGCCGATAGTGGAACCGACG
SeqDN	CCAGTTGCCTAAATGAACCATCTT
14/17RTF	AGCCGCGCTCACTCGGCACAAGA
14/17RTR	AACTCGGGTAGGTTACTATCGGC

*The restriction sites used are shown in italic type.

and pathogenicity in *M. oryzae*. This is the first demonstration that Pex14/17 plays a role in fungal pathogenicity.

The docking complex of peroxisomal matrix protein import is composed of Pex13 and Pex14 in all species analysed so far, whereas only yeasts appear to possess the additional component Pex17 (Huhse *et al.*, 1998; Kiel *et al.*, 2006). However, filamentous fungi, such as *M. oryzae*, lack Pex17 but possess Pex14/17. Mopex14/17 is a typical peroxisomally distributed protein and has properties of a hybrid protein composed of an N-terminal part sharing similarity with the Pex14 N-terminus and a C-terminal part sharing similarity to Pex17. These properties lead to the possibility that Mopex14/17 may act as a functional homologue of Pex17 or an alternative version of Pex14. Lack of Mopex14/17 leads to apparent disorders of the import of peroxisomal matrix proteins and typical defects in peroxisome-related metabolism and development, such as lipid utilization, ROS resistance and pathogenicity. These results imply that Mopex14/17 plays a role in peroxisome biogenesis and is not redundant to Pex14. To confirm this point, we undertook a cross-complement experiment and found that neither *MoPEX14* nor *MoPEX14/17* could restore the defects in the deleted mutants of each other (unpublished data). In addition, the N-terminal fraction of Pex14/17 in *P. chrysogenum* was unable to complement the defects of $\Delta pex14/17$. These facts indicate that Pex14/17 differs from Pex14 in function, despite the structural similarity. In *S. cerevisiae*, Pex17 contains a Pex19 binding site, and the peroxisomal targeting and stability of Pex17 require Pex19, the chaperone and/or receptor for integral PMPs. *MoPEX19* deletion alters the peroxisomal distribution of Mopex14/17 to a cytoplasmic distribution, indicating that the import of Pex14/17, similar to that of Pex17, relies on *PEX19*-dependent machinery. In addition, the N-terminally truncated Pex14/17 is able to complement both the matrix protein import and sporulation defects of $\Delta pex14/17$ of *P. chrysogenum*, indicating that the Pex17-related portion is crucial for the function of Pex14/17 as a peroxin. These findings indicate that Pex14/17 may function in a similar manner to Pex17 in yeasts.

In *S. cerevisiae*, *PEX17* null mutant cells abolished the import of both PTS1- and PTS2-containing matrix proteins into peroxisomes, and even led to the absence of morphologically detectable peroxisomes (Huhse *et al.*, 1998). *Yarrowia lipolytica* *PEX17* plays a role in the import of a subset of matrix proteins into peroxisomes and the development of normal peroxisomes. In *Hansenula polymorpha*, although a significant mislocalization of matrix proteins was observed, the *PEX17*-deleted strain was able to form small import-competent peroxisomes. However, the absence of Mopex14/17 resulted in partial defects in the import of PTS1-containing proteins, but not PTS2-containing proteins. Meanwhile, the profile of peroxisomes described by GFP-PTS1 and GFP-PTS2 in the $\Delta mopex14/17$ mutant was similar to that in the wild-type in terms of number, size and distribution. Such a phenomenon

could be observed in the Pex14/17 mutant of *P. chrysogenum* (Opaliński *et al.*, 2010). These results indicate that the absence of Pex14/17 leads to partial mislocalization of peroxisomal matrix proteins, but does not affect the presence of peroxisomal structures, and, in this sense, fungal *PEX14/17* is probably less influential to peroxisomal biogenesis than *PEX17* in yeasts.

As the core components of the peroxisomal docking complex for the PTS receptors, Pex13 and Pex14 are required for translocation of both PTS1- and PTS2-containing proteins across the peroxisomal membrane via interaction with the PTS receptors in humans, yeast and *Arabidopsis* (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould *et al.*, 1996). In the filamentous fungus *C. orbiculare*, the *PEX13* deletion mutants are incapable of the import of peroxisome matrix proteins possessing either a PTS1 or PTS2 targeting signal, and both conidia and appressorium-like structures formed by the $\Delta copex13$ mutant contain no visible peroxisomes (Fujihara *et al.*, 2010). GFP-PTS1 is completely diffused in the cytosol in $\Delta pex13$ and $\Delta pex14$ of *Po. anserina* (Peraza-Reyes *et al.*, 2011). The *PEX14* deletion strain of *N. crassa* is also severely defective in the sublocalization of peroxisomal matrix proteins (Managadze *et al.*, 2007). Disruption of *PEX13* and *PEX14* in *M. oryzae* gave similar results (unpublished data). Comparatively, *PEX14/17* is likely to have a lesser impact on peroxisomal import than the other two components of the docking complex, as its disruption only led to partial inhibition of the PTS1 pathway in *M. oryzae*, as well as in *P. chrysogenum* and the vegetative hyphae of *Po. anserina* (Opaliński *et al.*, 2010; Peraza-Reyes *et al.*, 2011). These findings indicate that Pex14/17, as a peripheral peroxin, may participate in peroxisomal matrix protein import by assisting Pex13 and Pex14. However, in *N. crassa*, the absence of *PEX14/17* resulted in more severe defects, where the PTS1-containing proteins were almost completely mislocalized and the peroxisomal density was severely decreased, as in $\Delta ncpx14$ mutants (Managadze *et al.*, 2007). More interestingly, *Po. anserina* *PEX14/17* was found to be differentially required for peroxisome assembly in the different phases of meiotic development (Opaliński *et al.*, 2010). We also compared the impacts of *MoPEX14/17* on peroxisomal import in different developmental stages of *M. oryzae*, but found no significant variation. These facts reflect the functional differences in Pex14/17, even between closely related species. Further work is needed to unravel the detailed mechanism of Pex14/17 and its interactions with other functional members in peroxisomal import.

To date, seven *PEX* genes, *MoPEX5*, *MoPEX6*, *MoPEX7*, *MoPEX19*, *MoPEX11A*, *MoPEX11B* and *MoPEX11C*, have been characterized in *M. oryzae* (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008, 2015). The absence of *MoPEX5* disables the PTS1 import pathway, *MoPEX6* plays a vital role in the import of both PTS1- and PTS2-containing proteins,

and *MoPEX19* is indispensable for the translocation of not only matrix proteins, but also PMPs (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008). The disruption of the three genes leads to more severe damage to matrix protein import, as the PTS1 proteins are completely cytosolic in the mutants of these genes, in contrast with the partial mislocation in $\Delta mopex14/17$. Correspondingly, the absence of these genes leads to more severe defects in peroxisomal metabolism than does the absence of *MoPEX14/17*. For instance, the growth of the mutants is entirely inhibited on olive oil or Tween as sole carbon source, whereas $\Delta mopex14/17$ shows only reduced growth with a normal colony appearance.

As a model for plant fungal pathogens, the rice blast fungus *M. oryzae* has been demonstrated to use peroxisomal metabolism in its invasive processes (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008, 2015). The roles of peroxisomes in fungal pathogenicity are generally related to lipid degradation, ROS elimination, and appressorial melanization and turgor generation. The $\Delta mopex14/17$ strain causes reduced symptoms on rice and barley, confirming that the peroxisomes are essential for plant infection with *M. oryzae*. However, the virulence reduction in $\Delta mopex14/17$ is apparently lower than that in $\Delta mopex5$, $\Delta mopex6$ or $\Delta mopex19$, which show a complete loss of pathogenicity (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008). Accordingly, $\Delta mopex5$, $\Delta mopex6$ and $\Delta mopex19$ are deficient in appressorial melanization and turgor genesis (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008), whereas $\Delta mopex14/17$ only lacks appressorial turgor. Meanwhile, the defects resulting from the deletion of *MoPEX14/17* in colony morphology, conidial genesis, ROS resistance and cell wall integrity are all generally less severe than those in $\Delta mopex5$, $\Delta mopex6$ or $\Delta mopex19$ (Li *et al.*, 2014; Ramos-Pamplona and Naqvi, 2006; Wang *et al.*, 2007, 2008). Therefore, the contributions of these peroxins to pathogenicity correspond well with their performance in peroxisomal biogenesis, metabolism and pathogenicity-related development, and pathogenicity is thus a good indicator of the roles of peroxisomal biogenesis genes and the status of peroxisomal metabolism in *M. oryzae*.

In summary, we have functionally characterized *PEX14/17*, encoding a fungal-specific peroxin probably as an integral part of the peroxisomal docking complex, in peroxisomal biogenesis, fungal development and pathogenicity in *M. oryzae*. We demonstrate that *MoPEX14/17* plays an essential role in the import of PTS1 proteins and, as a consequence, participates in lipid degradation, ROS elimination and cell wall integrity. The disruption of *MoPEX14/17* leads to a series of defects in colony morphology, appressorial formation and turgor generation, and, as a result, significantly reduces the virulence of the fungus to its host plants. All the primers used in this work were listed in Table 1.

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REFERENCES

- Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J.A., Veenhuis, M. and Kunau, W.H. (1997) Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. *Cell*, **89**, 83–92.
- Asakura, M., Okuno, T. and Takano, Y. (2006) Multiple contributions of peroxisomal metabolic function to fungal pathogenicity in *Colletotrichum lagenarium*. *Appl. Environ. Microbiol.* **72**, 6345–6354.
- Bartoszewski, M., Opaliński, L., Veenhuis, M. and van der Klei, I.J. (2011) The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotechnol. Lett.* **33**, 1921–1931.
- Bonnet, C., Espagne, E., Zickler, D., Boissard, S., Bourdais, A. and Berteaux-Lecellier, V. (2006) The peroxisomal import proteins PEX2, PEX5 and PEX7 are differently involved in *Podospora anserina* sexual cycle. *Mol. Microbiol.* **62**, 157–169.
- Braverman, N., Steel, G., Obie, C., Moser, A., Moser, H., Gould, S.J. and Valle, D. (1997) Human PEX7 encodes the peroxisomal PTS2 receptor and is responsible for rhizomelic chondrodysplasia punctata. *Nat. Genet.* **15**, 369–376.
- Brocard, C. and Hartig, A. (2006) Peroxisome targeting signal 1: is it really a simple tripeptide? *Biochim. Biophys. Acta*, **1763**, 1565–1573.
- Brocard, C., Kragler, F., Simon, M.M., Schuster, T. and Hartig, A. (1994) The tetrapeptide repeat domain of the PAS10 protein of *Saccharomyces cerevisiae* is essential for binding the peroxisomal targeting signal-SKL. *Biochem. Biophys. Res. Commun.* **204**, 1016–1022.
- Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997) Pex14p is a member of the protein linkage map of Pex5p. *EMBO J.* **16**, 5491–5500.
- Crawford, M.S., Chumley, F.G., Weaver, C.G. and Valent, B. (1986) Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. *Genetics*, **114**, 1111–1129.
- Dean, R.A., Talbot, N.J., Ebbole, D.J., Farman, M.L., Mitchell, T.K., Orbach, M.J., Thon, M., Kulkarni, R., Xu, J.R. and Pan, H. (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature*, **434**, 980–986.
- Distel, B., Erdmann, R., Gould, S.J., Blobel, G., Crane, D.I., Cregg, J.M., Dodt, G., Fujiki, Y., Goodman, J.M. and Just, W.W. (1996) A unified nomenclature for peroxisome biogenesis factors. *J. Cell Biol.* **135**, 1–3.
- Dodt, G. and Gould, S.J. (1996) Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell Biol.* **135**, 1763–1774.
- Ebbole, D.J. (2007) *Magnaporthe* as a model for understanding host–pathogen interactions. *Annu. Rev. Phytopathol.* **45**, 437–456.
- Elgersma, Y., Kwast, L., Klein, A., Voorn-Brouwer, T., Van Den Berg, M., Metzger, B., America, T., Tabak, H.F. and Distel, B. (1996) The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import PTS1-containing proteins. *J. Cell Biol.* **135**, 97–109.
- Erdmann, R. and Blobel, G. (1996) Identification of Pex13p a peroxisomal membrane receptor for the PTS1 recognition factor. *J. Cell Biol.* **135**, 111–121.
- Fan, J., Quan, S., Orth, T., Awai, C., Chory, J. and Hu, J. (2005) The Arabidopsis PEX12 gene is required for peroxisome biogenesis and is essential for development. *Plant Physiol.* **139**, 231–239.
- Faust, P.L., Banka, D., Siriratsivawong, R., Ng, V.G. and Wikander, T.M. (2005) Peroxisome biogenesis disorders: the role of peroxisomes and metabolic dysfunction in developing brain. *J. Inherit. Metab. Dis.* **28**, 369–383.
- Fujihara, N., Sakaguchi, A., Tanaka, S., Fujii, S., Tsuji, G., Shiraishi, T., O'Connell, R. and Kubo, Y. (2010) Peroxisome biogenesis factor PEX13 is required for appressorium-mediated plant infection by the anthracnose fungus *Colletotrichum orbiculare*. *Mol. Plant–Microbe Interact.* **23**, 436–445.
- Glover, J.R., Andrews, D.W. and Rachubinski, R.A. (1994) *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA*, **91**, 10 541–10 545.
- Goh, J., Jeon, J., Kim, K.S., Park, J., Park, S.Y. and Lee, Y.H. (2011) The PEX7-mediated peroxisomal import system is required for fungal development and pathogenicity in *Magnaporthe oryzae*. *PLoS One*, **6**, e28220.

- Gould, S.J., Keller, G.A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **108**, 1657–1664.
- Gould, S.J., Kalish, J.E., Morrell, J.C., Bjorkman, J., Urquhart, A.J. and Crane, D.I. (1996) Pex13p is an SH3 protein of the peroxisome membrane and a docking factor for the predominantly cytoplasmic PTS1 receptor. *J. Cell Biol.* **135**, 85–95.
- Heath, M.C., Valent, B., Howard, R.J. and Chumley, F.G. (1990) Interactions of two strains of *Magnaporthe grisea* with rice, goosegrass, and weeping lovegrass. *Can. J. Bot.* **68**, 1627–1637.
- Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, N.P. (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA*, **88**, 11 281–11 284.
- Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K. and Kunau, W.H. (1998) Pex17p of *Saccharomyces cerevisiae* is a novel peroxin and component of the peroxisomal protein translocation machinery. *J. Cell Biol.* **140**, 49–60.
- Hynes, M.J., Murray, S.L., Khew, G.S. and Davis, M.A. (2008) Genetic analysis of the role of peroxisomes in the utilization of acetate and fatty acids in *Aspergillus nidulans*. *Genetics*, **178**, 1355–1369.
- Idnurm, A., Giles, S.S., Perfect, J.R. and Heitman, J. (2007) Peroxisome function regulates growth on glucose in the basidiomycete fungus *Cryptococcus neoformans*. *Eukaryot. Cell*, **6**, 60–72.
- Imazaki, A., Tanaka, A., Harimoto, Y., Yamamoto, M., Akimitsu, K., Park, P. and Tsuge, T. (2010) Contribution of peroxisomes to secondary metabolism and pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Eukaryot. Cell*, **9**, 682–694.
- Islinger, M., Cardoso, M. and Schrader, M. (2010) Be different—the diversity of peroxisomes in the animal kingdom. *Biochim. Biophys. Acta*, **1803**, 881–897.
- de Jong, J.C., McCormack, B.J., Smirnoff, N. and Talbot, N.J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244–244.
- Kankanala, P., Czymmek, K. and Valent, B. (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell*, **19**, 706–724.
- Kiel, J.A., Veenhuis, M. and van der Klei, I.J. (2006) PEX genes in fungal genomes: common, rare or redundant. *Traffic*, **7**, 1291–1303.
- Kimura, A., Takano, Y., Furusawa, I. and Okuno, T. (2001) Peroxisomal metabolic function is required for appressorium-mediated plant infection by *Colletotrichum lagenarium*. *Plant Cell*, **13**, 1945–1957.
- Lazarow, P.B. (2003) Peroxisome biogenesis: advances and conundrums. *Curr. Opin. Cell Biol.* **15**, 489–497.
- Lazarow, P.B. (2006) 1. 7. The import receptor Pex7p and the PTS2 targeting sequence. *Biochim. Biophys. Acta*, **1763**, 1599–1604.
- Li, L., Wang, J., Zhang, Z., Wang, Y., Liu, M., Jiang, H., Chai, R., Mao, X., Qiu, H. and Liu, F. (2014) MoPex19, which is essential for maintenance of peroxisomal structure and Woronin bodies, is required for metabolism and development in the rice blast fungus. *PLoS One*, **9**, e85252.
- Managadze, D., Würtz, C., Sichtung, M., Niehaus, G., Veenhuis, M. and Rottensteiner, H. (2007) The peroxin PEX14 of *Neurospora crassa* is essential for the biogenesis of both glyoxysomes and Woronin bodies. *Traffic*, **8**, 687–701.
- Managadze, D., Würtz, C., Wiese, S., Schneider, M., Girzalsky, W., Meyer, H.E., Erdmann, R., Warscheid, B. and Rottensteiner, H. (2010) Identification of PEX33, a novel component of the peroxisomal docking complex in the filamentous fungus *Neurospora crassa*. *Eur. J. Cell Biol.* **89**, 955–964.
- McNew, J.A. and Goodman, J.M. (1994) An oligomeric protein is imported into peroxisomes in vivo. *J. Cell Biol.* **127**, 1245–1257.
- Min, K., Son, H., Lee, J., Choi, G.J., Kim, J.C. and Lee, Y.W. (2012) Peroxisome function is required for virulence and survival of *Fusarium graminearum*. *Mol. Plant–Microbe Interact.* **25**, 1617–1627.
- Nottingham, J. and Silue, D. (1992) Distribution of the mating type alleles in *Magnaporthe grisea* populations pathogenic on rice. *Phytopathology*, **82**, 421–424.
- Nuttall, J.M., Motley, A. and Hetttema, E.H. (2011) Peroxisome biogenesis: recent advances. *Curr. Opin. Cell Biol.* **23**, 421–426.
- Opaliński, Ł., Kiel, J.A., Homan, T.G., Veenhuis, M. and van der Klei, I.J. (2010) *Penicillium chrysogenum* Pex14/17p—a novel component of the peroxisomal membrane that is important for penicillin production. *FEBS J.* **277**, 3203–3218.
- Peraza-Reyes, L., Zickler, D. and Berteaux-Lecellier, V. (2008) The peroxisome RING-finger complex is required for meiosis formation in the fungus *Podospora anserina*. *Traffic*, **9**, 1998–2009.
- Peraza-Reyes, L., Arnaise, S., Zickler, D., Coppin, E., Debuchy, R. and Berteaux-Lecellier, V. (2011) The importomer peroxins are differentially required for peroxisome assembly and meiotic development in *Podospora anserina*: insights into a new peroxisome import pathway. *Mol. Microbiol.* **82**, 365–377.
- Pieuchot, L. and Jedd, G. (2012) Peroxisome assembly and functional diversity in eukaryotic microorganisms. *Annu. Rev. Microbiol.* **66**, 237–263.
- Ramos-Pamplona, M. and Naqvi, N.I. (2006) Host invasion during rice-blast disease requires carnitine-dependent transport of peroxisomal acetyl-CoA. *Mol. Microbiol.* **61**, 61–75.
- Rho, H.S., Kang, S. and Lee, Y.H. (2001) *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. *Mol. Cells*, **12**, 407–411.
- Rucktäschel, R., Halbach, A., Girzalsky, W., Rottensteiner, H. and Erdmann, R. (2010) De novo synthesis of peroxisomes upon mitochondrial targeting of Pex3p. *Eur. J. Cell Biol.* **89**, 947–954.
- Saikia, S. and Scott, B. (2009) Functional analysis and subcellular localization of two geranylgeranyl diphosphate synthases from *Penicillium paxilli*. *Mol. Genet. Genom.* **282**, 257–271.
- Stehlik, T., Sandroock, B., Ast, J. and Freitag, J. (2014) Fungal peroxisomes as biosynthetic organelles. *Curr. Opin. Microbiol.* **22**, 8–14.
- Swinkels, B.W., Gould, S., Bodnar, A., Rachubinski, R. and Subramani, S. (1991) A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.* **10**, 3255.
- Talbot, N.J. (2003) On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. *Annu. Rev. Microbiol.* **57**, 177–202.
- Talbot, N.J., Ebbole, D.J. and Hamer, J.E. (1993) Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell*, **5**, 1575–1590.
- Tzafir, I., Pena-Muralla, R., Dickerman, A., Berg, M., Rogers, R., Hutchens, S., Sweeney, T.C., McElver, J., Aux, G. and Patton, D. (2004) Identification of genes required for embryo development in Arabidopsis. *Plant Physiol.* **135**, 1206–1220.
- Van der Leij, I., Franse, M.M., Elgersma, Y., Distel, B. and Tabak, H.F. (1993) PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*, **90**, 11 782–11 786.
- Walton, P.A., Hill, P. and Subramani, S. (1995) Import of stably folded proteins into peroxisomes. *Mol. Biol. Cell*, **6**, 675–683.
- Wanders, R.J.A. and Waterham, H.R. (2005) Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. *Clin. Genet.* **67**, 107–133.
- Wanders, R.J. and Waterham, H.R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem.* **75**, 295–332.
- Wang, J., Wu, X., Zhang, Z., Du, X., Mao, X. and Sun, G. (2008) Fluorescence localization of PTS1 and PTS2 in PEX5 and PEX7 mutants of *Magnaporthe grisea*. *J. Biotechnol.* **136**, S221.
- Wang, J., Zhang, Z., Wang, Y., Li, L., Chai, R., Mao, X., Jiang, H., Qiu, H., Du, X. and Lin, F. (2013) PTS1 peroxisomal import pathway plays shared and distinct roles to PTS2 pathway in development and pathogenicity of *Magnaporthe oryzae*. *PLoS One*, **8**, e55554.
- Wang, J., Li, L., Zhang, Z., Qiu, H., Li, D., Fang, Y., Jiang, H., Chai, R.Y., Mao, X. and Wang, Y. (2015) One of three Pex11 family members is required for peroxisomal proliferation and full virulence of the rice blast fungus *Magnaporthe oryzae*. *PLoS One*, **10**, e0134249.
- Wang, Z.Y., Soanes, D.M., Kershaw, M.J. and Talbot, N.J. (2007) Functional analysis of lipid metabolism in *Magnaporthe grisea* reveals a requirement for peroxisomal fatty acid β -oxidation during appressorium-mediated plant infection. *Mol. Plant–Microbe Interact.* **20**, 475–491.
- Wilson, R.A. and Talbot, N.J. (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nat. Rev. Microbiol.* **7**, 185–195.