Fusarium verticillioides FvPex8 Is a Key Component of the Peroxisomal Docking/Translocation Module That Serves Important Roles in Fumonisin Biosynthesis but Not in Virulence

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Peroxisomes are ubiquitous organelles in eukaryotes that fulfill various important metabolic functions. In this study, we investigated the role of docking/translocation module (DTM) peroxins, mainly FvPex8, FvPex13, FvPex14, and FvPex33, in *Fusarium verticillioides* development, virulence, and fumonisin B₁ (FB₁) biosynthesis. Protein interaction experiments suggested that FvPex13 serves as the central DTM subunit in *F. verticillioides*. Notably, FvPex8 and FvPex14 did not show direct interaction in our experiments. We generated gene-deletion mutants (Δ Fvpex8, Δ Fvpex13, Δ Fvpex14, Δ Fvpex33, Δ Fvpex33/14) and further examined the functional role of these peroxins. Deletion mutants exhibited disparity in carbon nutrient utilization and defect in cell-wall integrity when stress agents were applied. Under nutrient starvation, mutants also showed higher levels of lipid droplet accumulation. Particularly, Δ Fvpex8 mutant showed significant FB₁ reduction and altered expression of key FB₁ biosynthesis genes. However, FvPex13 was primarily responsible for asexual conidia reproduction and virulence, while the Δ Fvpex33/14 double mutant also showed a virulence defect. In summary, our study suggests that FvPex13 is the central component of DTM, with direct physical interaction with other DTM peroxins, and regulates peroxisome membrane biogenesis as well as PTS1- and PTS2-mediated transmembrane cargo transportation. Importantly, we also characterized FvPex8 as a key component in *F. verticillioides* DTM that affects peroxisome function and FB₁ biosynthesis.

Keywords: docking/translocation module, fumonisin B₁, *F. verticillioides*, peroxins, virulence

Peroxisomes are a family of membrane-bound microbodies found in all major eukaryotes, with specific metabolic properties showing substantial differences among various species and tissue types (Alberts et al. 2002; Cooper 2000). These cellular organelles, including specialized peroxisomes called glyoxysomes in plants and fungi and glycosomes in trypanosomes, are involved in a variety of important cellular functions, including fatty acid metabolism, reactive oxygen species (ROS) detoxification, and secondary metabolite biosynthesis (Chen et al. 2018; Imazaki et al. 2010; Maggio-Hall et al. 2005; Reverberi et al. 2012; Saikia and Scott 2009). Dysfunctional peroxisomes have been linked to severe and lethal disorders in humans and mammals (Cho et al. 2018; Dixit et al. 2010; Thoms et al. 2009; You et al. 2015). Plant glyoxysomes play essential roles in glyoxylate cycle, embryonic development, and gametophytic functions (Boisson-Dernier et al. 2008; Hu et al. 2012). Glycosomes in trypanosomes perform important glycolytic reactions (Bauer and Morris 2017). Published studies also suggest that peroxisomes play important roles in development, secondary metabolism, and virulence in plant-pathogenic fungi (Kimura et al. 2001; Soundararajan et al. 2004; Wang et al. 2013), but underlying molecular mechanisms associated with these fungal-specific functions still warrant further investigation.

Fusarium verticillioides is a major pathogen of maize, particularly due to ear rot disease and mycotoxin contamination worldwide (Marin et al. 2013; Woloshuk and Shim 2013; Wu et al. 2014). Interestingly, a recent study described a key peroxisomal matrix protein (FVEG_11334, which is designated as FvPex33 in this study) and a Woronin body-associated protein (FvHex1) interacting with Fsr1, a striatin homolog that regulates virulence in *F. verticillioides* (Zhang et al. 2018b). While our preliminary study revealed that FvPex33 and FvHex1 do not directly influence *F. verticillioides* virulence when tested on maize seedlings, earlier published studies on fungal peroxins (Fujihara et al. 2010; Min et al. 2012; Li et al. 2017) suggested that key peroxisomal proteins play important roles in *F. verticillioides* virulence and mycotoxin biosynthesis.

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Biogenesis of peroxisomes is dynamically regulated in response to environmental and developmental cues (Heiland and Erdmann 2005; Lazarow 2003). Metabolic tasks and enzyme content are adjusted according to taxa-specific cellular needs through generation and degradation (Nuttall et al. 2011; Pieuchot and Jedd 2012). There are two primary pathways for peroxisome formation: de novo biogenesis and mature peroxisomes fission. The de novo synthesis of peroxisomes has two steps: formation of the peroxisomal membrane and subsequent sorting of peroxisomal matrix proteins to the organelle (Opaliński et al. 2011). The peroxisomal importomer model helps explain how newly synthesized matrix proteins bind to the cytosolic receptor at the peroxisome membrane and form a highly dynamic pore to transfer matrix proteins to peroxisome lumen (Meinecke et al. 2010). These transient cargo-docking-translocation complexes are formed by a variable combination of peroxin proteins (Pex) (Supplementary Table S1). Briefly, the key docking complex core consists of the SH3-domain protein Pex13 and the PXXP-motif protein Pex14 but also harbors additional factors, e.g., Pex17 in lower eukaryotic cells, Pex33 (also known as Pex14/17) in filamentous fungi, and Pex13.2 in *Trypanosoma brucei* (Verplaetse et al. 2012). A recent study also characterized a Pex17/Pex14/Dyn2p complex in yeast (Chan et al. 2016). Physical association among docking peroxins varies in different organisms, and published reviews extensively explain how various matrix proteins with type 1 and type 2 peroxisomal targeting signal (PTS1 and PTS2) are recognized by the specific docking complex and associate with the peroxisomal membrane (Nuttall et al. 2011; Pieuchot and Jedd 2012).

PTS1 and PTS2 peroxisomal matrix proteins are recognized by specific receptors Pex5 and Pex7, respectively, and recruited to the peroxisome docking complex (Heiland and Erdmann 2005; Meinecke et al. 2010). In addition to these core peroxins in the docking/translocation module (DTM), other peroxins are dynamically involved in translocating matrix proteins across the peroxisome membrane (Platta et al. 2013). In particular, Pex8 is known to be associated with DTM but is only present in yeasts and filamentous fungi (Agne et al. 2003). It has been suggested that Pex8 can serve as an importomer-bridging protein to hold the docking complex and the export machinery complex together to facilitate dynamic and transient translocation (Meinecke et al. 2010; Platta et al. 2013; Rayapuram and Subramani 2006), but the detailed function of this peroxin is largely unknown. In this study, our aim was to characterize putative *F. verticillioides* peroxins, particularly those predicted to be involved in DTM. While paying close attention to two core components of the docking complex, Pex13 and Pex14, we expanded our study into additional peroxisomal proteins, namely Pex8, that have not been well-characterized in plant-pathogenic fungi. Through a series of fungal metabolism, physiology, and virulence assays, we concluded that Pex8, along with Pex13, plays a critical role in the generation and function of DTM that ultimately impacts *F. verticillioides* virulence and mycotoxin biosynthesis.

RESULTS

Protein-protein interactions between *F. verticillioides* DTM components and other key peroxins.

Peroxis Pex13 and Pex14 are recognized as key peroxisomal membrane docking complex proteins in eukaryotes, which are required for protein import into peroxisomes. In addition, Pex33 has been identified as another component of the docking complex in *Neurospora crassa* (Managadze et al. 2010), and Pex8 is known to be associated with DTM in yeasts and filamentous fungi (Agne et al. 2003). Using these proteins as query, we screened for putative DTM components in *F. verticillioides*, i.e., FvPex8, FvPex13, FvPex14, and FvPex33, using the BLASTP

algorithm (Supplementary Table S1). Additional details on these key peroxins, e.g., sequence similarities and putative functional motifs, are provided in Supplementary Table S1 and Supplementary Fig. S1A. Notably, further comparison of FvPex33 against Pex14- and Pex17-like proteins in *Saccharomyces cerevisiae*, *Fusarium graminearum*, and *F. verticillioides* strongly suggested that FvPex33 is distinct from Pex14 and Pex17 and is a unique DTM component in *F. verticillioides* (Supplementary Fig. S1B). Based on the prediction of transmembrane regions and orientations (TMpred) algorithm (Hoffman and Stoffel 1993), FvPex8, FvPex13, FvPex14, and FvPex33 are predicted to have transmembrane helices and likely belong to peroxisomes membrane proteins (Supplementary Fig. S1C).

To study the cellular localization of these proteins in *F. verticillioides*, we first chose the biogenesis protein FvPex14 as the peroxisomal marker and prepared FvPex14-mCherry construct (Grant et al. 2013). We studied transformants containing green fluorescent protein (GFP) fused to FvPex8, FvPex13, or FvPex33 protein along with FvPex14-mCherry for overlapping red and green fluorescence signals (Fig. 1A), and these images revealed that FvPex14 colocalizes with FvPex13 and FvPex33 while showing only partial overlap with FvPex8. To further verify physical interactions between these DTM subunits, we coexpressed the following six pairs of proteins, i.e., FvPex13-GFP/FvPex14-mCherry, FvPex13-GFP/FvPex8-mCherry, FvPex13-GFP/FvPex33-Flag, FvPex33-GFP/FvPex14-mCherry, FvPex8-GFP/FvPex33-Flag, and FvPex8-GFP/FvPex14-mCherry, into *F. verticillioides* for a coimmunoprecipitation (co-IP) experiment. Results showed that FvPex13-GFP successfully associated with the beads and that FvPex14 was eluted in samples that coexpressed FvPex13-GFP and FvPex14-mCherry, indicating FvPex13 and FvPex14 binding in vivo (Fig. 1B). Similarly, FvPex13 successfully associated with FvPex8 and FvPex33 (Fig. 1C and D). FvPex33 successfully associated with FvPex14 and FvPex8 (Fig. 1E and F), but FvPex8 did not coprecipitate with FvPex14 (Fig. 1G). We further tested these interactions by yeast two-hybrid assays (Supplementary Fig. S2A), in which FvPex13 showed strong positive interaction with key DTM proteins tested. FvPex8 showed strong interaction with FvPex13 but not with FvPex14. Based on these outcomes, we propose that FvPex13 serves as the central subunit in *F. verticillioides* DTM. Furthermore, these protein interaction studies provide clearer evidence that FvPex8 and FvPex33 are important components of DTM along with FvPex13 and FvPex14. However, the interaction between FvPex8 and FvPex14 is deemed indirect and conceivably mediated by FvPex13.

Vegetative growth and conidiation are differentially affected by carbon sources in DTM peroxin mutants.

To study the function of key DTM peroxins in *F. verticillioides*, we generated Δ Fvpex8, Δ Fvpex13, Δ Fvpex14, and Δ Fvpex33 through targeted gene replacement with the hygromycin-resistance marker in *F. verticillioides* 7600 wild-type (WT) strain, as described previously (Zhang et al. 2018b). We also generated a Δ FvPex33/14 double mutant by replacing the *FvPEX14* gene with the geneticin-resistance marker in Δ Fvpex33 protoplast. Subsequently, we also generated complementation strains Δ Fvpex8-C, Δ Fvpex13-C, Δ Fvpex14-C, and Δ Fvpex33-C by transforming the corresponding mutant with a construct harboring the gene (with its native promoter and terminator) fused with GFP and the geneticin resistance marker (*GEN*). Homologous recombination was confirmed by PCR of targeted loci and by quantitative PCR (qPCR) of gene expression in corresponding knockout and complementation strains (Supplementary Fig. S3).

To test whether these mutations impact *F. verticillioides* growth on various carbon nutrients, we cultivated these strains

Fig. 1. FvPex8, FvPex13, FvPex14, and FvPex33 protein interaction analyses. **A**, Localization of FvPex8-GFP (green fluorescent protein), FvPex13-GFP, and FvPex33-GFP were studied in comparison with FvPex14-mCherry, which was used as a peroxisomal marker, in the wild-type strain. Scale bar = 10 μ m. **B**, GFP trap-based pull-down experiment showing the interaction in vitro between FvPex13 and FvPex14. **C**, FvPex13 and FvPex8. **D**, FvPex33 and FvPex13. **E**, FvPex33 and FvPex14. **F**, FvPex33 and FvPex8, and **G**, FvPex8 and FvPex14. The strains coexpressing the indicated proteins were immunoprecipitated with GFP trap beads. The immunoprecipitation (IP) signal (FvPex13-GFP, FvPex8-GFP, or FvPex33-GFP) and the co-IP signal (FvPex8-mCherry, FvPex14-mCherry, or FvPex33-Flag) were detected by immunoblots probed with antibodies to GFP, mCherry, and Flag as indicated.

on minimum medium (MM) and complete (CMII) agar plates amended with different carbon sources. On MM agar, we observed varying growth inhibition rates and colony morphologies with different carbon nutrient (Fig. 2A). No strain showed a particularly detrimental growth defect on MM, suggesting that these genes are not critical for vegetative growth. But on MM agar with sucrose, vegetative growth and aerial mycelia production in $\Delta Fvpex8$ and $\Delta Fvpex33/14$ was notably hindered (Fig. 2A and B). The inhibition rate was more drastic in $\Delta Fvpex8$ on MM agar with butyrate and palmitic acid (Fig. 2A and B). Interestingly, aerial mycelium was completely absent in the $\Delta Fvpex8$ mutant on media with palmitic acid. On CMII agar with sucrose as the carbon source, only $\Delta Fvpex8$ exhibited slight growth inhibition, but, overall, all strains were morphologically very similar (Supplementary Fig. S4A). On CMII with linoleic acid, growth inhibition in $\Delta Fvpex8$ was more drastic compared with other mutants (Supplementary Fig. S4B).

Redox homeostasis, cell-wall integrity, and lipid degradation in DTM mutants.

Peroxisomes serve as one of the primary sources of endogenous and exogenous ROS production. Accumulation of ROS and disruption of redox homeostasis can lead to lower cell survival (Min et al. 2012). We cultured strains on liquid MM, and, when stained with CM-H₂DCFDA (6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate), mutant hyphae showed green fluorescent granules indicative of high ROS accumulation, especially in $\Delta Fvpex8$, $\Delta Fvpex13$, and $\Delta Fvpex33/14$ mutants (Fig. 3A and B). The colonies were stained with 3,3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) for H₂O₂ and O₂⁻ detection, respectively. Yellow and blue colonies were observed, in $\Delta Fvpex8$, $\Delta Fvpex13$, and $\Delta Fvpex33/14$ mutants, with DAB and NBT staining, respectively (Fig. 3C). Peroxin mutants are likely to display cell-wall integrity defects due to their inability to import enzymes of the glyoxylate cycle into peroxisomes (Bhambra et al. 2006). Almost all mutants exhibited sensitivity to

Calcofluor white except for the $\Delta Fvpex8$ mutant (Fig. 4A). Mutants showed much higher sensitivity in general to sodium dodecyl sulfate (SDS) when compared with the WT (Fig. 4A). We also observed growth inhibition in DTM mutants on medium amended with H₂O₂ when compared with the WT, which suggested that the ability to respond to ROS and maintain redox homeostasis was disturbed in these peroxin mutants (Fig. 4A). The growth inhibition rate in peroxin mutants was significantly higher than in the WT, suggesting DTM peroxins are important for maintaining *F. verticillioides* cell-wall integrity (Fig. 4B). We also studied lipid droplet degradation and turnover defects in DTM mutants. The mutants accumulated higher levels of lipid droplets compared with the WT, particularly when shifted to nutrient starvation conditions (Supplementary Fig. S5).

DTM peroxin FvPex8 function is necessary for fumonisin B₁ (FB₁) production but not in asexual reproduction in *F. verticillioides*.

F. verticillioides is one of the key fungal pathogens responsible for fumonisin contamination of maize worldwide. To assess FB₁ accumulation, we independently inoculated B73 maize kernels with spore suspensions of WT, $\Delta Fvpex8$, $\Delta Fvpex13$, $\Delta Fvpex14$, $\Delta Fvpex33$, and $\Delta Fvpex33/14$ strains. After incubation for 10 days, fungal mass in samples was assessed by qPCR quantification of the β -tubulin gene (*TUB2*). Fungal mass in $\Delta Fvpex8$, $\Delta Fvpex13$, and $\Delta Fvpex33/14$ mutants was approximately half those observed in WT, $\Delta Fvpex14$, and $\Delta Fvpex33$ strains (data not shown). When FB₁ levels were normalized to fungal mass in each sample, all mutants showed reduction in FB₁ production, ranging from 50 to 94%, with $\Delta Fvpex8$ showing the most drastic reduction to less than 10% when compared with WT samples (Fig. 5A).

Published studies suggest close genetic association between secondary metabolite production and asexual reproduction in filamentous fungi (Adams and Yu 1998). The fact that all peroxin mutants showed significant reduction in FB₁, especially in

Fig. 2. Impact of FvPex8 on carbon source utilization. **A**, Vegetative growth and aerial mycelia production in wild type (WT) and mutant strains were monitored on minimal medium (MM) amended with different carbon sources. Colony morphology was photographed after a 5-day incubation at 25°C. **B**, The growth inhibition rate was analyzed by comparing the growth of WT with mutants on MM agar with various carbon amendments. The mycelial growth inhibition rate (%) was measured by (the diameter of WT strain – the diameter of mutant strain)/(the diameter of WT strain) × 100%. Bar indicates the standard deviation of three replicates.

Δ Fvpex8, we harvested conidia from strains grown on CMII agar plates after 5 days of incubation. As seen in Figure 5B, conidia production in mutants was negatively impacted in all mutants tested. However, the conidiation production defect was most drastic in Δ Fvpex13 and Δ Fvpex33/14 mutants.

Fig. 3. Disruption of peroxisomal functions promotes reactive oxygen species (ROS) accumulation. **A**, Fungal hyphae were grown for 2 days on complete (CMII) medium and were stained with 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) to measure cellular ROS accumulation in the wild-type (WT) strain and mutants. Accumulation of ROS in mycelia was visualized CM-H₂DCFDA staining. Scale bar = 10 μ m. **B**, ROS accumulation was quantified by Image J software. Error bars represent the deviation from three replicates and one or two asterisks above the column indicates a statistically significant difference ($P < 0.05$ or < 0.01 , respectively) when compared against WT, as analyzed by t test. **C**, Endogenous ROS scavenging capacity was tested by staining fungal strains grown on CMII agar for 5 days with 3,3'-diaminobenzidine (DAB) for H₂O₂ detection and with nitro blue tetrazolium (NBT) for O₂⁻ detection.

These outcomes suggest that, while FvPex8 plays a key role in FB₁ biosynthesis, FvPex13 serves as a key regulatory factor in asexual reproduction in *F. verticillioides*.

DTM peroxin FvPex13 plays key roles in *F. verticillioides* virulence.

To test whether these DTM peroxins are associated with virulence, we inoculated B73 maize stalk internodes with spore suspensions of WT, Δ Fvpex8, Δ Fvpex13, Δ Fvpex14, Δ Fvpex33, and Δ Fvpex33/14 strains. When stalks were split open longitudinally after a 10-day incubation, Δ Fvpex13 and Δ Fvpex33/14 mutants showed drastically reduced rot when compared with the WT progenitor and other mutants (Fig. 5C and D). While Δ Fvpex14 and Δ Fvpex33 mutants did not impact stalk rot virulence, the double mutant Δ Fvpex33/14 showed a significant reduction in virulence. Significantly, the Δ Fvpex8 mutant did not exhibit any negative effect on *F. verticillioides* virulence. The FvPex13-C strain showed WT-level stalk rot symptoms, suggesting that FvPex13 plays a critical role in *F. verticillioides* virulence on maize and sugarcane.

Deletion of Pex13 impacts cellular localization of DTM peroxins in *F. verticillioides*.

A mutation in Pex13 has been linked to PTS1 import defects in yeast as well as peroxisome abnormalities and appressorium defects in *Colletotrichum orbiculare* (Erdmann and Blobel 1996; Fujihara et al. 2010). To monitor PTS matrix protein import in *F. verticillioides*, we used confocal microscopy to follow GFP derivatives targeted to the peroxisome. We fused GFP to carnitine *O*-acetyl transferase gene FvCAT containing C-terminal PTS1 sequence (Zolman and Bartel 2004) and peroxisomal 3-oxoacyl-CoA thiolase gene FvFOX3 containing N-terminal PTS2 sequence (Einerhand et al. 1995), thus obtaining fusion protein GFP-PTS1 and PTS2-GFP, respectively. We also tested physical interactions between these two PTS proteins and PTS receptor proteins by yeast two-hybrid assays, and FvPex5 and FvPex7 showed positive results with FvCat and FvFox3, respectively (Supplementary Fig. S2B and C). GFP-PTS1 showed a diffused fluorescence distributed throughout the cytoplasm, while PTS2-GFP was restricted to few subcellular organelles in cytoplasm of 2-day-old Δ Fvpex13 hyphae (Fig. 6A). Pex14-mCherry is shown as the peroxisomal marker. These results led us to hypothesize that FvPex13 plays a role in peroxisomal protein targeting via its interaction with FvPex5 and FvPex7. We learned that PTS1 receptor FvPex5 predominantly localizes to the cytoplasm, very similar to FvCat, whereas PTS2 receptor FvPex7 and mPTS receptor FvPex19 are mostly found in cytoplasm in Δ Fvpex13 mutants (Fig. 6B). For FvPex8, we performed FM4-64 staining study to get further evidence that FvPex8 is not associated with vacuoles in *F. verticillioides* (Supplementary Fig. S6). These results suggested that FvPex13 function is not only required to maintain localization but also affect the steady-state size and number levels of these docking peroxins affecting peroxisomes.

DISCUSSION

The key aim of this study was to gain a deeper understanding of how peroxisomal DTM complex impacts peroxin import, the integrity and functionality of peroxisomes, and ultimately FB₁ biosynthesis and virulence in maize pathogen *F. verticillioides*. Interaction between four DTM peroxins, i.e., FvPex8, FvPex13, FvPex14, and FvPex33, were demonstrated through co-IP and yeast two-hybrid assays. Interestingly, FvPex8 exhibited no direct interaction with FvPex14. We also confirmed that FvPex14-mCherry colocalizes with FvPex13-GFP, FvPex8-GFP, and FvPex33-GFP in mycelia, suggesting that these proteins form a complex in *F. verticillioides*. FvPex13 plays a more predominant

role in translocating proteins with PTS1 than PTS2, though FvPex13 is interactive with both PTS receptors. In *S. cerevisiae*, Pex14 preferentially binds to the cargo-loaded Pex5 receptor for pore formation while Pex13 is coupled to the translocation of a cargo protein, and the close association of Pex13 and Pex14 is required for peroxisomal matrix protein import (Schell-Steven et al. 2005). It is also suggested that Pex13 serves as the initial binding site for the PTS2 preimport complex, whereas Pex14 assembles with other peroxins in high-molecular weight complexes after or during dissociation of the PTS2 receptor (Grunau et al. 2009; Meinecke et al. 2010). Based on literature and our experimental data, we envisaged a schematic model of *F. verticillioides* peroxisomal DTM complex (Fig. 7). This is an important finding, since the functional role of Pex8 and its association with other key DTM components has not been characterized in fungal pathogens previously.

Published studies provide numerous examples how key DTM proteins Pex13 and Pex14 are important not only for peroxisome biogenesis but also for a host of other cellular and physiological functions in filamentous fungi. Absence of the docking peroxin Pex13 in *Podospira anserina* obstructed sexual development prior to meiosis (Suaste-Olmos et al. 2018). *C. orbiculare* Pex13 is required for appressorium-mediated plant infection, namely for melanin biosynthesis and turgor generation (Fujiyama et al. 2010). Chen and colleagues (2018) showed that Pex13 also plays an important role in *F. graminearum* pathogenesis and mycotoxin biosynthesis. We found very similar outcomes in our study, where *F. verticillioides* FvPex13 mediated plant infection and severe peroxisomal defects. Pex14, in addition to its key function as a docking protein, plays a main role in the formation of two separate import pores, facilitating PTS1- and PTS2-dependent protein imports into the peroxisomal matrix (Meinecke et al. 2010; Montilla-Martinez et al. 2015). Pex14 has a unique dual function in peroxisome formation and in pexophagy in *Pichia pastoris* and *F. graminearum* (Bellu et al. 2001; Chen et al. 2018; Deng et al. 2013; Farré et al. 2008; Lee et al. 2017). Pex14 serves an important role in Woronin body formation in *Neurospora crassa* and host infection in *F. graminearum* (Chen et al. 2018; Managadze et al. 2007). In addition to Pex13 and Pex14, another important component of the DTM complex in filamentous fungi is Pex33, a chimeric protein consisting of an N-terminal Pex14-like domain and a C-terminal

Pex17-like domain. Published studies have linked Pex33 with glyoxysome biogenesis, sexual and asexual development, virulence, and secondary metabolism (Chen et al. 2018; Li et al. 2017; Managadze et al. 2010; Opaliński et al. 2010; Peraza-Reyes et al. 2011). In our study, we found *F. verticillioides* FvPex14 and FvPex33 collaborate to mediate plant infection and partial peroxisomal functions.

While these key Pex proteins have been extensively investigated in filamentous fungi and other eukaryotes, the role of Pex8 in peroxisomal import machinery and its impact on other physiological roles in pathogenic fungi are still unclear. Pex8, originally discovered as Per1 protein in yeast *Hansenula polymorpha* (syn. *Pichia angusta*), is an intraperoxisomal peroxin that has a PTS1 at the C-terminus and an unusual PTS2 in the mid-protein region (Agne et al. 2003; Waterham et al. 1994). Pex8 is currently the only recognized intraperoxisomal peroxin, while the majority of peroxins are either peroxisomal membrane-bound or in the cytosol (Girzalsky et al. 2010; Ma et al. 2009; Pieuchot and Jedd 2012). Pex8 is targeted to peroxisomes by cytosolic receptors Pex5 (PTS1) or Pex7 (PTS2), and its membrane translocation is primarily mediated through DTM Pex14 (Agne et al. 2003; Ma et al. 2009). Once positioned in the peroxisomal matrix, Pex8 becomes an important component of the importomer complex, anchoring key receptors as well as other membrane-bound peroxins, and serves an important function in both PTS1- and PTS2-dependent protein translocation into peroxisome (Agne et al. 2003; Rehling et al. 2000). Therefore, it is important to acknowledge that composition and organization of the importomer and docking complex can vary in eukaryotes, not only in a species-dependent manner but also in a cargo-dependent manner (Gabaldón 2010; Heiland and Erdmann 2005; Lazarow 2003).

In our study, we learned that FvPex8 plays a crucial role in *F. verticillioides* secondary metabolism but is dispensable for plant pathogenesis. Involvement of peroxisomes in fungal secondary metabolite biosynthesis has been well-documented, including in penicillin, AK toxin, paxilline, and deoxynivalenol (Chen et al. 2018; Imazaki et al. 2010; Martín et al. 2012; Herr and Fischer 2014; Saikia and Scott 2009). However, it is still unclear how FvPex8 impacts the FB₁ biosynthetic pathway or regulation. We learned that the expression of key *FUM* genes is decreased drastically in Δ FvPex8 mutants, but it is highly

Fig. 4. Cell wall and H₂O₂ stress response in mutants. **A**, Mycelial growth of the wild type (WT) and mutant strains was monitored on complete (CMII) media supplemented with sodium dodecyl sulfate (SDS), Calcofluor white (CFW), and H₂O₂ for 5 days. Plates were photographed after a 5-day incubation at 25°C. **B**, Mycelial growth inhibition rates were calculated by (the diameter of WT strain – the diameter of mutant strain)/(the diameter of WT strain) × 100%.

unlikely FvPex8 directly regulates genes in the *FUM* cluster. However, it is possible that Pex8, which contains both peroxisomal targeting signals PTS1 and PTS2, functions as an organizer of the peroxisomal protein import machinery. In *S. cerevisiae*, Pex8 is located at the inner surface of the peroxisomal membrane and is essential for the assembly of the importomer (Deckers et al. 2010). Acetyl-CoA, one of the key building blocks for the synthesis of polyketides, can be produced via various catabolic pathways, including peroxisomal β -oxidation of fatty acids (Maggio-Hall et al. 2005). The Δ Fvpex8 mutant showed difficulty growing on multiple carbon sources, raising the question whether FvPex8 plays a role in peroxisomal carbon recycling and β -oxidation functions. When investigating *F. verticillioides* PTS proteins *in silico*, we also learned that palmitoyl-CoA hydrolase (FVEG_02271, designated FvAct) contains PTS1 and serine palmitoyl transferase (FVEG_12143, designated FvPat) contains PTS2. We postulate that FvPex8 indirectly impacts hydrolysis of palmitoyl-CoA, which is an acyl-CoA thioester that plays a role in biosynthesis

of sphingolipids and in transport of fatty acyl-CoA molecules into the mitochondria for β -oxidation (Brady et al. 1969). Based on current DTM models, inner membrane FvPex8 function may impact both import receptors FvPex5 (PTS1 cargo shuttle) and FvPex7 (PTS2 cargo shuttle) and disrupt import and export of acetyl-CoA necessary for secondary metabolism in *F. verticillioides*. While we observed higher expression levels of *FvPEX5* and *FvPEX7* when we performed retrograde gene regulation of *FvPEX* genes in Δ Fvpex8 (Supplementary Results), further study is needed to test this hypothesis.

One unexpected outcome in our study was Δ Fvpex8 showing no particular defect in virulence. Studies have shown how key peroxisome biogenesis genes are associated with plant infection and pathogenesis in *Colletotrichum lagenarium*, *C. orbiculare*, *Magnaporthe grisea*, *Alternaria alternata*, and *F. graminearum* (Fujihara et al. 2010; Goh et al. 2011; Imazaki et al. 2010; Min et al. 2012; Ramos-Pamplona and Naqvi 2006; Wang et al. 2007). For instance, CoPex13 is required for appressorium melanization and turgor generation, which are essential for

Fig. 5. Role of peroxins in fumonisin B₁ (FB₁) production, asexual reproduction, and maize stalk rot virulence. **A**, Surface-sterilized B73 corn kernels were inoculated with wild-type (WT) and mutant conidia and were incubated for 10 days before FB₁ levels were quantified by an enzyme-linked immunosorbent assay kit. *Fusarium verticillioides* biomass was quantified by measuring *F. verticillioides* β -tubulin (*TUB2*) DNA in samples. The relative FB₁ level in samples was measured by calculating FB₁ (parts per million)/*TUB2* DNA (nanograms). **B**, To compare the asexual reproduction capacity in WT and mutant strains, conidia production in WT and mutant strains was measured after incubation on complete (CMII) agar at room temperature for 8 days. **C**, Maize B73 stalks were inoculated with WT and mutant fungal spore suspension (10^6 /ml) at the internodal region and were incubated at room temperature for 7 days. Subsequently, maize stalks were split longitudinally to visually inspect rot symptoms. Sterile water was used as a negative control with no rot symptom observed after incubation (data not shown). **D**, Image J software was used to quantify the extent of the rot in each sample. All experiments were performed in three independent biological repetitions and error bars represent the standard deviation from three replicates. One or two asterisks ($P < 0.05$ or < 0.01 , respectively) above the column indicate a statistically significant difference when compared against WT as analyzed by *t* test. Sterile water was used as a negative control (A, B, and D).

appressorium penetration ability in *C. orbiculare* (Fujihara et al. 2010). MoPex33 is required for the import of peroxisomal matrix proteins and full virulence in *M. oryzae* (Li et al. 2017). A recent publication described how Pex13, Pex14, and Pex33 play a critical role against host stress agents and pexophagy, ultimately impacting fungal virulence in *F. graminearum* (Chen

et al. 2018). Along with these key DTM components, import receptors Pex5 and Pex7 were also shown to be important for fitness and virulence in fungal pathogens (Goh et al. 2011; Min et al. 2012; Wang et al. 2013; Zhang et al. 2018a). FvPex8 showed strong physical interaction with FvPex13, therefore it was reasonable for us to hypothesize that FvPex8 also plays a

Fig. 6. Fvpex13 plays an important role in mycelial peroxin localization. **A**, Localization of green fluorescent protein (GFP) fused to C-terminal PTS1 sequence (PTS1-GFP) and N-terminal PTS2 sequence (GFP-PTS2) were studied in the wild type (WT) and Δ Fvpex13 mutant. FvPex14-mCherry signal was used as the peroxisomal marker. Bars = 2 μ m. **B**, Localization of key docking/translocation module peroxins FvPex8, FvPex7, FvPex19, and FvPex5 were studied in WT and the Δ Fvpex13 mutant. In addition, FvPex14-GFP was monitored in Δ Fvpex14 and Δ Fvpex13, while FvPex33-GFP was monitored in Δ Fvpex33 and Δ Fvpex13.

critical role in pathogenesis. However, this was not the case. Only $\Delta FvPex13$ showed impaired virulence in *F. verticillioides* (Fig. 5C). FvPex13 is known to affect efficient PTS1 and PTS2 imports, and this requires FvPex5-FvPex13 interaction in vivo (Pires et al. 2003; Schell-Steven et al. 2005). Loss of peroxisome function may slow pathogen growth due to inefficient utilization of carbon sources from the host and the fungus itself (Fig. 2; Supplementary Fig. S4). However, we did not observe similar response in the host when challenged by the $\Delta Pex8$ mutant, which suggested that FvPex8, while it forms a complex with FvPex13 and other DTM components, may not directly contribute to virulence in *F. verticillioides*.

To summarize, we identified and characterized *F. verticillioides* homologs of DTM components FvPex8, FvPex13, FvPex14, and FvPex33 in this study. Based on our experimental outcomes and literature, we proposed a schematic model of core *F. verticillioides* DTM complex (Fig. 7). It is reasonable to hypothesize that these proteins are not only involved in the regulation of peroxisomal docking complex assembly and matrix translocation but also ultimately impact various aspects of fungal development, secondary metabolism, and virulence. We propose that the DTM complex plays important roles in virulence and asexual reproduction through FvPex13, likely in association with FvPex14 and FvPex33. For FB₁ production, FvPex8 was determined as the key component, but the mechanism in which FvPex8 regulates FB₁ biosynthesis needs further investigation.

MATERIALS AND METHODS

Fungal strains and culture media.

F. verticillioides WT strain 7600 and all transformants (Supplementary Table S2) were cultured on CMII (2% sucrose, 0.05% magnesium sulfate heptahydrate, 0.1% potassium dihydrogen phosphate, 0.05% potassium chloride, 0.2% nitrate potassium acid, 0.1% hydrolyzed casein, 0.2% peptone, 0.1% Wolfe's Vitamin Solution [Coolaber], 1.8% agar) and MM (Leslie and Summerell 2006). CMII agar with 250 μ g of hygromycin B (Roche) per milliliter, 200 μ g of G418 (Sigma) per milliliter, or

800 μ g of G418 (Sigma) per milliliter were used for screening transformants. For conidia production assay, six 0.7-cm diameter agar blocks were added to 3 ml of water and were vortexed and conidia were quantified by a hemocytometer. For vegetative growth and stress agent sensitivity assays, the WT and mutant strains were grown on MM and CMII amended with various carbon sources: potassium acetate (40 mM), sodium butyrate (10 mM), sucrose (2%), palmitic acid (3 mM), oleic acid (3 mM), or linoleic acid (3 mM) (Min et al. 2012). Colony diameter was measured after a 5-day incubation. Cell-wall integrity was tested by growing the strains on CMII supplemented with 200 μ g of Calcofluor white (CFW) per milliliter or 0.01% SDS. The tolerance of strains to exogenous ROS was evaluated by measuring growth on CMII containing 0.05% H₂O₂.

Generation of the DTM gene-deletion mutants and GFP-, mCherry-, or Flag-tagged strains.

Single DTM gene *F. verticillioides* mutants were generated with a split-marker approach described previously (Zhang et al. 2018b). All primers used in this study are listed in Supplementary Table S3. For double deletion of *FvPEX14* and *FvPEX33*, we replaced *FvPEX14* with the neomycin phosphotransferase (*NEO*) gene in the $\Delta FvPex33$ mutant. Protoplasts were generated, transformed, and screened following our standard PCR and qPCR (Yan and Shim 2020; Zhang et al. 2018b). For qPCR, the WT and DTM mutants were inoculated in CMII medium for 3 days. The extraction of total RNA and the synthesis of first-strand cDNA were performed as previously described (Yan and Shim 2020). The expression of peroxins was detected by qPCR, using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech). As an endogenous control, a 200-bp amplicon of *F. verticillioides* β -tubulin (*TUB2*) gene (FVEG_04081) was amplified, and the relative quantification of each transcript was calculated following the standard $2^{-\Delta\Delta CT}$ method previously used (Yan and Shim 2020). Primers used to amplify selected genes in qPCR reactions are listed in Supplementary Table S3. All qPCR assays were conducted in triplicates for each sample and the experiment was repeated three times. Subsequently, we constructed the fusion GFP and mCherry vectors following the protocol described previously (Yan and Shim 2020; Zhang et al. 2018b). All fusion vectors generated were sequenced before transformation. For construction of GFP-tagged strains, we transformed corresponding fusion GFP vectors with a G418 sulfate-resistant (*GEN*) gene into protoplasts. For testing whether FvPex13 affected the localization of PTS pathway-related genes, we transformed fusion GFP vectors with *GEN* into WT and $\Delta FvPex13$ mutant protoplasts. For testing whether FvPex33 interacts with other DTM, we transformed Flag vectors and fusion Flag-FvPex33 with *GEN* into WT and $\Delta FvPex33$ mutant protoplasts, respectively.

ROS and lipid staining and quantification.

For lipid staining, 2-day-old mycelia were harvested as 0 h, and corresponding 2-day-old mycelia were transferred to phosphate buffered saline (PBS) medium for 18 h, washing mycelia twice with water. Hypha was tested by staining with Nile Red at 0 and 18 h, respectively (Nguyen et al. 2011). Cellular ROS accumulation in the fungal hyphae was analyzed using CM-H₂DCFDA staining. Mycelia were harvested and washed with PBS three times. Hypha was stained with CM-H₂DCFDA (5 μ M) (Sigma) for 10 min, in the dark, following GENMED reagent protocols and scanning confocal microscope with excitation at 490 nm (GENMED Scientifics). Accumulation of ROS in mycelia was visualized with CM-H₂DCFDA staining. Endogenous ROS scavenging capacity was tested by growing the strains on MM agar for 5 days, and the colony was stained with

Fig. 7. Proposed schematic model of *Fusarium verticillioides* peroxisome docking/translocation module (DTM). This proposed model is based on our protein-protein interaction experiments as well as other published fungal models. The model shows FvPex13, FvPex14 (dimer), FvPex8, and FvPex33 forming the core DTM module. PTS1-specific receptor FvPex5 and PTS2-specific receptor FvPex7 work closely with the core DTM module to recruit PTS1- and PTS2-peroxisomal matrix proteins (PTS1-Cat, PTS1-FvAct, PTS2-Fox, and PTS2-FvPat), which are subsequently translocated into peroxisomes as depicted by red dotted arrows. Our study determined distinct role each component plays, particularly, FvPex13 being the central component in regulating virulence, likely in close association with FvPex14 and FvPex33, and FvPex8 playing a prominent role in *F. verticillioides* mycotoxin biosynthesis.

DAB for H₂O₂ detection and with 2 mM NBT (Sigma) in 20 mM phosphate buffer, pH 6, for O₂⁻ detection.

Co-IP and yeast two-hybrid assays.

Strains were grown in CMII medium for 2 days before immunoblot and IP analyses were conducted, as previously described (Zheng et al. 2015). For total protein extraction, mycelia of FvPex13-GFP and FvPex14-mCherry were ground into a fine powder in liquid nitrogen and were resuspended in 30 ml of extraction buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride). The supernatants were centrifuged 15,000 × g for 25 min at 4°C to remove cell debris. For IP of GFP-fusion proteins from cellular extracts, equal concentrations of total proteins were isolated and incubated with 20 to 30 µl of GFP-Trap agarose beads (ChromoTek), according to the manufacturer instructions. Proteins eluted from GFP-Trap agarose beads were analyzed by immunoblot detection with the anti-mCherry, anti-Flag (Clontech), and anti-GFP (Sigma) antibodies.

The yeast two-hybrid assay was carried out following our previous standard protocol (Zhang et al. 2018b). The coding sequence of each tested gene was amplified from WT cDNA. *FvPEX13* and *FvPEX14* were cloned into pGBKT7 as the bait vectors. Other components of the DTM *FvPEX8*, *FvPEX14*, *FvPEX33*, along with *FvPEX5* and *FvPEX7* cDNA were amplified and cloned into pGADT7 as the prey vectors. Plasmid pairs pGBKT7-P53/pGADT7-T and pGBKT7-Lam/pGADT7-T were used as the positive and the negative control, respectively. All primers used in this study are listed in Supplementary Table S3. Further details are provided in Supplementary Information.

Virulence and FB₁ assays.

Infection assays on maize stalks were conducted as previously described (Zhang et al. 2015; Zhang et al. 2018b). Subsequently, maize stalks were split longitudinally to assess disease severity and to quantify the extent of the rot, using Image J software. For FB₁ assay, conidia were inoculated on surface-sterilized B73 corn kernels for 10 days and were quantified with a FB₁ enzyme-linked immunosorbent assay kit, following manufacturer-suggested protocol (Biovision). FB₁ concentrations were obtained by the optical density of each well at 450 nm, with a microplate reader, by comparing to the FB₁ standard curve. *F. verticillioides* biomass quantification was calculated by qPCR based on the *TUB2* standard curve, which was measured at concentrations of 0.01, 0.1, 1.0, 10 ng/µl (Rocha et al. 2016). Sample FB₁ levels were normalized by calculating the FB₁ parts per million/*TUB2* DNA concentration.

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