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Deletion of *PdMit1*, a homolog of yeast *Csg1*, affects growth and Ca^{2+} sensitivity of the fungus *Penicillium digitatum*, but does not alter virulence

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Abstract

GDP-mannose:inositol-phosphorylceramide (MIPC) and its derivatives are important for Ca^{2+} sensitization of *Saccharomyces cerevisiae* and for the virulence of *Candida albicans*, but its role in the virulence of plant fungal pathogens remains unclear. In this study, we report the identification and functional characterization of *PdMit1*, the gene encoding MIPC synthase in *Penicillium digitatum*, one of the most important pathogens of postharvest citrus fruits. To understand the function of *PdMit1*, a *PdMit1* deletion mutant was generated. Compared to its wild-type control, the *PdMit1* deletion mutant exhibited slow radial growth, decreased conidia production and delayed conidial germination, suggesting that *PdMit1* is important for the growth of mycelium, sporulation and conidial germination. The *PdMit1* deletion mutant also showed hypersensitivity to Ca^{2+} . Treatment with 250 mmol/l Ca^{2+} induced vacuole fusion in the wild-type strain, but not in the *PdMit1* deletion mutant. Treatment with 250 mmol/l CaCl_2 upregulated three Ca^{2+} -ATPase genes in the wild-type strain, and this was significantly inhibited in the *PdMit1* deletion mutant. These results suggest that *PdMit1* may have a role in regulating vacuole fusion and expression of Ca^{2+} -ATPase genes by controlling biosynthesis of MIPC, and thereby imparts *P. digitatum* Ca^{2+} tolerance. However, we found that *PdMit1* is dispensable for virulence of *P. digitatum*.

Keywords

Penicillium digitatum; GDP-mannose:inositol-phosphorylceramide; *PdMit1*; Ca^{2+} sensitivity; Ca^{2+} -ATPase

Conflict of interest

We declare that we have no conflict of interest.

1. Introduction

Sphingolipids are ubiquitous lipid components of eukaryotes, including filamentous fungi. In addition to the structural components of membranes, sphingolipids and their metabolites are now recognized as important bioactive molecules that modulate various cellular events, including cell proliferation, differentiation, endocytosis, apoptosis, nutrient uptake, calcium homeostasis and the stress response [1,2]. Fungal sphingolipids are distinct from those found in plants, animals and humans, and the enzymes responsible for synthesis of fungus-specific sphingolipids may serve as potential targets of antifungal drugs [3,4].

Biosynthesis of sphingolipids has been intensively studied in *Saccharomyces cerevisiae* [5,6]. It starts with condensation of palmitoyl-CoA and serine in the endoplasmic reticulum [7]. After undergoing a series of reactions, including reduction, hydroxylation and acylation, phytoceramide is synthesized [8]. Thereafter, IPC synthase encoded by *Aur1* transfers a phosphoryl-inositol moiety from phosphatidylinositol to the 1-hydroxy group of phytoceramide to form IPC (inositol-phosphorylceramide) [9]. IPC is further modified by the addition of mannose to form MIPC (mannose inositol-phosphorylceramide), and then another inositol phosphate group is further introduced to MIPC by inositol phosphotransferase to form M(IP)₂C. In the plasma membrane, IPC, MIPC, M(IP)₂C and other complex sphingolipids constitute the lipid rafts with cholesterol and sterols [10]. Lipid rafts have been elaborated to participate in assembly of the subcortical actin cytoskeleton and are involved in material transport and endocytosis [11,12]. Two distinct MIPC synthase complexes, *Csg1* (calcium sensitive gene)-*Csg2* and *Csh1* (*Csg1* homolog)-*Csg2*, participate in the addition of mannose to the inositol 2-OH moiety of IPC to form MIPC in *S. cerevisiae*. The subunits encoded by *Csg1* and *Csh1* perform catalysis and the subunit encoded by *Csg2* performs a regulatory function [13].

In *S. cerevisiae*, the absence of both *Csg1* and *Csg2* resulted in a significant reduction in MIPC. Accumulation of IPC-C was also observed in this double gene deletion mutant [14]. It was reported that *Csg1* and *Csg2* play an important role in Ca²⁺ tolerance in *S. cerevisiae*. Moderate concentrations (about 100 mmol/l) of Ca²⁺ could induce death of the *Csg1* and *Csg2* double deletion mutant [15]. A *Csg1* homolog deletion mutant of *Candida albicans*, *Aspergillus fumigatus* and *Schizosaccharomyce pombe* also exhibited increased Ca²⁺ sensitivity [14,16–18]. When accumulated IPC-C was converted into IPC-D, the precursor of MIPC, Ca²⁺ sensitivity of the MIPC synthase-deficient mutant was suppressed, suggesting that the accumulation of IPC-C is associated with Ca²⁺ sensitivity [15,18].

MitA in *Aspergillus fumigatus* was the first *Csg1* homolog to be characterized in filamentous fungi. In the *MitA* deletion mutant of *A. fumigatus*, IPCs accumulated, whereas M(IP)₂C was absent due to a lack of transfer of mannose to IPCs [18]. Conidia germination of the *MitA* deletion mutant of *A. fumigatus* was decelerated under normal growth conditions, but the virulence of this mutant was not impaired [18].

Mit1, the homolog of *S. cerevisiae* *Csg1*, was also required for formation of MIPC-derived complex sphingolipids and phospholipomannan in *C. albicans* [17]. A previous study found that β-mannosylation of phospholipomannan played a role in the virulence of *C. albicans* by

contacting host cells and diffusing into the cell wall [19]. Consistently, the *Mit1* deletion mutant of *C. albicans* was less virulent during both the acute and chronic phases of systemic infection in mice due to reduced β -mannosylation of phospholipomannan [17].

Citrus green mold caused by *Penicillium digitatum* is the most destructive disease of postharvest citrus fruits and causes significant loss during post-harvest storing, packaging, transportation and marketing of citrus fruits [20]. Despite its economic importance, the molecular mechanisms involved in growth, sporulation, adaptation to the changing environment and pathogenesis of *P. digitatum* have not been fully elucidated. In this study, we report the identification of *PdMit1*, an ortholog of *Csg1/Mit1* from *P. digitatum*, and we demonstrate that *PdMit1* is important for mycelial growth, sporulation and conidial germination of *P. digitatum*, but not for virulence.

2. Materials and methods

2.1. Fungal strains and culture conditions

Wild-type strain PdKH8 of *P. digitatum* used in this study was collected from Kaihua County, Zhejiang Province, China in 2010. Both the wild-type strain and its derivative mutant strains were cultured on PDA (potato dextrose agar) at 25 °C. Conidia were obtained by scraping the agar surface with sterile distilled water at 5 dpi (days post inoculation).

2.2. Sequence analysis of *PdMit1*

To identify the putative gene encoding MIPC synthase in *P. digitatum*, the amino acid sequences of *S. cerevisiae* Csg1p/Sur1p (systematic name, YPL057C), Csh1p (YBR161W) and Csg2p (YBR036C) [15,21] were used as queries in BLASTp search of the *P. digitatum* genome [22]. Sequences of DNA and cDNA of *PdMit1* were amplified from the genomic DNA and cDNA of wild-type strain PdKH8, respectively, with primers Mit1-F/Mit1-R (Table S1). The amplified fragments were cloned into the pMD18-T vector and sequenced. The putative protein of *PdMit1*p was predicted for subcellular localization using TargetP 1.1 (www.cbs.dtu.dk/services/TargetP/). SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) and TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) were used for the analysis of transmembrane domains (TMDs) and the location of signal peptide cleavage sites, respectively.

2.3. Construction of the *PdMit1* deletion mutant

Two flanking sequences of the *PdMit1* gene amplified from *P. digitatum* genomic DNA were inserted upstream and downstream of the *hph* (hygromycin resistance) gene of the pTFCM vector, respectively. A 1439 bp fragment upstream of the *PdMit1* coding sequence was amplified with Mit1-up-F and Mit1-up-R (Table S1) and cloned into the *KpnI* and *SacI* sites of pTFCM vector to construct PTFCM-*PdMit1*-up. Then, a 1432 bp fragment downstream of the *PdMit1* coding sequence was amplified using primers Mit1-down-F and Mit1-down-R (Table S1) and inserted into the *SpeI* and *XhoI* sites of the pTFCM-*PdMit1*-up vector to generate *PdMit1* replacement vector pTFCM- *PdMit1*. This replacement vector was transformed into *Agrobacterium tumefaciens* AGL-1 by electroporation using ECM630 (BTX, CA, USA). To obtain the transformants, *A. tumefaciens*-mediated transformation

(ATMT) was performed as described previously [23]. The *PdMit1* deletion mutants were selected on PDA medium supplemented with 70 µg/ml hygromycin B. The identification of gene replacement mutants was performed by PCR using primers Mit1-check-F1/Mit1-check-R1 and Mit1-check-F2/Mit1-check-R2 (Table S1). Southern hybridization analysis was used to further confirm the *PdMit1* replacement. Genomic DNA of the *PdMit1* deletion mutant or wild-type strain was cut with the *EcoRII* and *BamHI* restriction enzymes, respectively. The probe (787 bp) corresponding to the upstream flanking sequence of the *PdMit1* gene was amplified from genomic DNA of the wild-type strain with the primers Mit1-SB-F and Mit1-SB-R (Table S1). DIG high prime DNA (Roche, Mannheim, Germany) was used for Southern blot following the protocol of the manufacturer.

2.4. Genetic complementation of the *PdMit1* deletion mutant

An approximate 4.2-kb full-length *PdMit1* gene, including the *PdMit1* coding region, its promoter (1.6-kb upstream of the start codon) and 0.9-kb sequence downstream of the stop codon was amplified from genomic DNA of wild-type strain PdKH8 using primers Mit1-com-F and Mit1-com-R (Table S1). This fragment was digested with *KpnI* and *SalI* and then cloned into plasmid pCA-neo to generate complementation vector pCA-neo-*PdMit1*. Transformation of the *PdMit1* deletion mutant with vector pCA-neo-*PdMit1* was conducted as described above and transformants were selected by neomycin. Identification of the *PdMit1* complement mutant (CPP*PdMit1*) was performed by PCR and Southern blot as described above.

2.5. Mycelial growth and conidiation assays

To determine the effect of *PdMit1* on mycelial growth, 5 µl of the conidial suspension (1.0×10^6 conidia/ml) of the *PdMit1* deletion mutant or wild-type strain PdKH8 were spotted separately on the center of PDA and CYA (Czapek yeast extract agar) plates. These plates were incubated at 25 °C. Radial growth was determined by calculating the mean of two perpendicular colony diameters in each plate at 4 dpi. Conidia were rinsed off the agar surfaces with sterile distilled water and conidial suspensions were diluted and counted in a hemocytometer at 5 dpi. The experiments were performed with three replicates.

2.6. Germination assays

One hundred microliters of the conidial suspension (1.0×10^4 conidia/ml) of the *PdMit1* deletion mutant or the wild-type strains were inoculated onto the surface of PDA medium, respectively. The germination of spores was observed at 8, 12, 16 and 24 h after incubation using an Eclipse 80i microscope (Nikon, Japan). When the germ tube extended to at least twice the length of the conidium, it was considered to be germinated. The germination rate (%) was defined as $[\text{germinated conidia}/(\text{germinated conidia} + \text{ungerminated conidia})] \times 100\%$. At least 100 conidia were assessed in a sample. The experiment was performed with three replicates.

2.7. Ion sensitivity assays

The sensitivity of the *PdMit1* deletion mutant and PdKH8 to ion stress was assessed by inoculating them individually on PDA medium supplemented with 300 mmol/l CaCl₂, 500

mmol/l NaCl, 750 mmol/l KCl, or 500 mmol/l MgSO₄. PDA medium with no ion supplement was used as a control. Each plate was inoculated with 5 µl of the conidial suspension (1.0×10^6 conidia/ml). The colony diameter was measured at 4 dpi. Growth inhibition was calculated as the percentage of radial growth in an ion-supplemented medium compared to that in the control plate. The experiments were repeated three times.

2.8. Amphotericin B sensitivity assays

For the assay of sensitivity to amphotericin B (AmB), a 5 µl conidial suspension (1.0×10^6 conidia/ml) of the *PdMit1* deletion mutant and PdKH8 were spotted onto PDA plates amended with 1 and 2 µg/ml AmB, respectively. Growth was scored at 4 dpi. Three replicates for each concentration were used for each strain.

2.9. Vacuole staining

A conidial suspension (100 µl, 1.0×10^7 conidia/ml) of the *PdMit1* deletion mutant or the wild-type strain of *P. digitatum* was added into PDB media (50 ml) supplemented with 250 mmol/l CaCl₂. After being incubated at 25 °C for 24 h, the young mycelia were collected by centrifugation at 6000 g for 5 min. The mycelia were then stained with neutral red as described [24] before vacuolar morphology was observed under an Eclipse 80i microscope (Nikon, Japan) equipped with a DS-Fi1 digital camera (Nikon, Japan).

2.10. Gene expression analysis of Ca²⁺-ATPases

To determine the expression levels of *PMCI* (accession number: JX298850), *PMR1* (accession number: JX298851) and *PMCA* (accession number: EKV07250), a 100 µl conidial suspension (1.0×10^7 conidia/ml) of the *PdMit1* deletion mutant or wild-type strain of *P. digitatum* was inoculated into PDB medium and cultured for 36 h at 25 °C with 180 rpm shaking. Then CaCl₂ was added to PDB medium with the final concentration of 250 mmol/l. After 6 h, total RNA was extracted from mycelia using an AxyPrep™ multisource total RNA miniprep kit (Axygen, China). Each sample was reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit with a gDNA eraser (TaKaRa, Dalian, China). The expression of these genes was determined using SYBR® Premix Ex Taq™ II on an Applied Biosystems 7500 Real-Time PCR system (ABI, Foster City, USA) according to the instructions. The β-actin gene (accession number: AB030227) was used as an internal control during the qRT-PCR. Primers used in this study were listed in Table S1. The relative changes in genes expression were analyzed according to the 2^{-Ct} method [25]. The experiment was conducted twice with three independent biological replicates. Data were calculated using analysis of t-test in SPSS version 13.0 for Windows (SPSS Inc. Chicago, IL, USA).

2.11. HPLC analysis of ergosterol

A conidial suspension (100 µl, 1.0×10^6 conidia/ml) of the *PdMit1* deletion mutant or the wild-type strains of *P. digitatum* was added into 100 ml PDB media and cultured at 25 °C for 2 days under shaking at 180 rpm. Mycelia were harvested and the ergosterol was extracted as described [26]. HPLC was carried out on a Waters alliance 2695 separation module (Waters, Milford, MA, USA) equipped with a Waters 2998 photodiode array

detection (Waters, Milford, MA, USA). Ergosterol was separated on Sunfire™ C18 column (250 mm × 4.6 mm, 5 µm particle size, Waters), using MeOH as the mobile phase. The flow rate was 1.5 ml/min. The detection wavelength was 282 nm. The identification of ergosterol was based on retention time. Determinations of ergosterol concentrations in samples were carried out by a calibration curve method using the peak height. The experiment was conducted twice with three independent biological replicates.

2.12. Virulence assay

Mature and unwounded Ponkan (*Citrus reticulata* Blanco) fruits were selected to investigate the role of *PdMit1* in *P. digitatum* virulence. Citrus peel was punctured (1–2 mm deep) with a bunch of 5 needles followed by inoculation with 5 ml of the conidial suspensions (1×10^6 conidia/ml) of the *PdMit1* deletion mutant or wild-type strain of *P. digitatum*. Sterile water was used as the negative control. These fruits were placed in a plastic tray and incubated at room temperature. The virulence of each strain was determined by the lesion diameter, which was measured at 4 dpi. Twenty fruits were used for each treatment and the experiment was repeated twice.

3. Results

3.1. *P. digitatum* expresses a putative MIPC synthase gene (*PdMit1*)

In *S. cerevisiae*, *Csg1*-, *Csg2*- and *Csh1* -constituted MIPC synthase complexes participated in mannosylation of IPC. A putative protein with the GenBank accession number EKV12295 from *P. digitatum* was found to be homologous to *S. cerevisiae* Csg1p and Csh1p, with 58% and 52% amino acid identity, respectively. This putative protein also shared 83% identity with *A. fumigatus* MitA. Interestingly, the homolog of *S. cerevisiae* Csg2p was not found in the *P. digitatum* genome. These observations prompted us to hypothesize that MIPC synthase involved in mannosylation of IPC in *P. digitatum* may be encoded by one gene, designated as *PdMit1*.

Sequencing analyses indicated that the sequence of *PdMit1* amplified from *P. digitatum* strain PdKH8 was completely identical to the DNA sequence encoding EKV12295 found in a Spanish strain Pd1 of *P. digitatum* [22]. *PdMit1* contained 1238 bp nucleotides with three introns, located at nt 280–331, nt 489–542 and nt 902–953 (started at ATG). The molecular mass of the protein product (*PdMit1p*) of the *PdMit1* was estimated at 41.77 kDa. A putative signal peptide was found at the N-terminal of PdMit1p with a cleavage site between position 22 and 23 (Fig. S1). There was also a catalytic region of 93 amino acid residues (positions 60–152) between two putative transmembrane segments (Fig. S1). This segment was homologous to the conserved domain of Och1p, a yeast α -1,6-mannosyltransferase. In this region, a DXD motif, which occurs in a wide range of glycosyltransferase families and likely has a role in the catalytic site [27], was also found.

3.2. The *PdMit1* deletion mutant and its complementation mutant were established

To study the function of *PdMit1*, *PdMit1* deletion mutants were created using a homologous recombination strategy as described in Materials and methods (Fig. S2A). Two transformants were identified as replacement mutants by PCR (Fig. S2B). Using the

internal primer pair Mit1-check-F1/Mit1-check-R1, a 501 bp fragment was amplified from the ectopic transformants, wild-type strain PdKH8 and the *CPPdMit1* mutant, but could not be amplified from *PdMit1* replacement mutants. On the other hand, with the outer primer pair Mit1-check-F2/Mit1-check-R2, a fragment of 2062 bp was amplified from the *PdMit1* deletion mutants and the *CPPdMit1* mutant, while no fragment could be amplified from the PdKH8 or ectopic transformants (Fig. S2B). The two *PdMit1* replacement mutants had identical phenotypes, including radial growth and Ca^{2+} sensitivity, so one replacement mutant, termed the *PdMit1* deletion mutant, was chosen for the later experiments. The *PdMit1* deletion mutant and *CPPdMit1* mutant were further confirmed by Southern blot. The probe specific to the 5' region of *PdMit1* was able to hybridize to 2263 bp and 1483 bp fragments the *PdMit1* deletion mutant and the wild-type strain of *P. digitatum*, respectively (Fig. S2C), confirming that the *PdMit1* deletion mutant was a null mutant resulting from a single homologous recombination event at the *PdMit1* locus.

3.3. *PdMit1* affects radial growth, sporulation and conidial germination in *P. digitatum*

The function of *PdMit1* on mycelial growth and sporulation was analyzed. The *PdMit1* deletion mutant exhibited reduced radial growth on both media (Fig. 1). The colony diameter of wild-type strain PdKH8 on PDA and CYA was 4.8 ± 0.2 cm and 3.5 ± 0.4 cm, respectively. Compared to wild-type strain PdKH8, the colony diameter of the *PdMit1* deletion mutant on PDA and CYA was reduced to 71% and 77%, respectively. Compared to wild-type strain PdKH8, sporulation of the *PdMit1* deletion mutant decreased. The amount of conidia for the *PdMit1* deletion mutant was 50% of the wild-type strain at 5 dpi (Fig. 2A). In addition, the germination percent of conidia also significantly declined for the *PdMit1* deletion mutant (Fig. 2B). The germination percent of conidia of the *PdMit1* deletion mutant was $17.9 \pm 4.2\%$ at 8 h after incubation, while more than half of the conidia for wild-type strain PdKH8 germinated at the same time. After incubation for 12 h, the germination percent of conidia of wild-type strain PdKH8 increased to $80.2 \pm 4.6\%$, but was $69.2 \pm 5.3\%$ for the *PdMit1* deletion mutant. At 16 h after incubation, near 100% of the conidia of the wild-type strain germinated, whereas 100% conidial germination of the *PdGcs1* deletion mutant occurred at 24 h.

Reintroduction of the *PdMit1* gene into the *PdMit1* deletion mutant resulted in the restoration of phenotypes in terms of its radial growth, sporulation and conidial germination percent (Figs. 1, 2A and 2B). These data suggest that *PdMit1* is involved in hyphal growth, sporulation and conidial germination in *P. digitatum*.

3.4. *PdMit1* decreases resistance of *P. digitatum* to Ca^{2+} stress

Sensitivity of the *PdMit1* deletion mutant and the wild-type strain of *P. digitatum* to ion stress was investigated by culturing them on PDA supplemented with CaCl_2 , MgSO_4 , NaCl and KCl (Fig. 3 and S3). Ca^{2+} at 100 mmol/l inhibited radial growth of the wild-type strain and the *PdMit1* deletion mutant by $6 \pm 3\%$ and $19 \pm 4\%$, respectively. Ca^{2+} at 300 mmol/l inhibited radial growth of the wild-type strain and the *PdMit1* deletion mutant by $28 \pm 5\%$ and $61 \pm 8\%$, respectively. Ca^{2+} at 400 mmol/l totally inhibited growth of the *PdMit1* deletion mutant, but did so only partially for the wild-type strain. The deletion of *PdMit1* also increased the sensitivity of *P. digitatum* to Mg^{2+} (Fig. S3). Compared to wild-type

strain PdKH8, radial growth of the *PdMit1* deletion mutant was more severely inhibited on PDA containing 500 mmol/l MgSO₄ (inhibition rate $35 \pm 4\%$ <mutant> vs. $8 \pm 2\%$ <wild-type>). Increased Ca²⁺ and Mg²⁺-sensitivities in the *PdMit1* deletion mutant were restored as *PdMit1* was introduced into the *PdMit1* deletion mutant (Figs. 3 and S3). However, the deletion of *PdMit1* did not affect the sensitivity of *P. digitatum* to Na⁺ and K⁺ (data not shown). These data suggest that *PdMit1* deletion sensitizes *P. digitatum* to growth inhibition by Ca²⁺ and Mg²⁺, but not Na⁺ and K⁺.

3.5. *PdMit1* regulates vacuole morphology

Vacuole morphology in both wild-type strain PdKH8 and the *PdMit1* deletion mutant was observed by microscopy after staining with neutral red. The morphology and distribution of vacuoles in mycelia of wild-type strain PdKH8 and the *PdMit1* deletion mutant cultured in normal PDB were similar (Fig. 4 A and B). However, when the PDB contained 250 mmol/l CaCl₂, the vacuole morphology in mycelia of the wild-type strain was significantly larger than that of the *PdMit1* deletion mutant and the number of vacuoles was significantly lower than in the *PdMit1* deletion mutant (Fig. 4C and D). The morphology and number of vacuoles in the *CPPdMit1* mutant were the same as in the wild-type strain (Fig. not shown). These results suggest that *PdMit1* regulates vacuole morphology and causes relative sensitivity of the *PdMit1* deletion mutant to high concentrations of Ca²⁺.

3.6. *PdMit1* affects expression of Ca²⁺-ATPase genes

The relative expression of three Ca²⁺-ATPase genes involved in Ca²⁺ transportation was compared between the wild-type strain and the *PdMit1* deletion mutant strain using real-time RT-PCR (Fig. 5). When they were transferred into PDB with 250 mmol/l CaCl₂, the expression of *PMCI* (vacuolar Ca²⁺-ATPase), *PMRI* (Golgi Ca²⁺-ATPase) and *PMCA* (plasma membrane Ca²⁺-ATPase) of wild-type strain PdKH8 increased to 2.6, 3.7 and 5.1 fold, respectively. However, compared to wild-type strain PdKH8, the expression of *PMCA* and *PMCI* in the *PdMit1* deletion mutant was not affected by 250 mmol/l CaCl₂. For *PMCA*, there was only slightly upregulation. When the *PdMit1* gene was reintroduced into the *PdMit1* deletion mutant, the expression levels of *PMRI*, *PMCA* and *PMCI* in the *CPPdMit1* mutant were the same as in wild-type strain PdKH8 (data not shown).

3.7. *PdMit1* affects sensitivity to AmB

A sensitivity test showed that the *PdMit1* deletion mutant was highly susceptible to polyene antifungal drug AmB (Fig. 6A). Growth of the *PdMit1* deletion mutant was significantly impaired on PDA containing AmB. AmB at 1 µg/ml inhibited radial growth of the *PdMit1* deletion mutant and the wild-type strain by $55 \pm 3\%$ and $34 \pm 5\%$, respectively. Growth of the *PdMit1* deletion mutant was virtually inhibited when the concentration of AmB reached 2 µg/ml, whereas wild-type strain PdKH8 and the *CPPdMit1* mutant still grew, suggesting that deletion of *PdMit1* increases the sensitivity of *P. digitatum* to AmB.

3.8. *PdMit1* affects the ergosterol level

The contents of ergosterol in wild-type strain PdKH8 and the *PdMit1* deletion mutant were 1.17 ± 0.1 and 1.41 ± 0.06 mg/g, respectively (Fig. 6B). The difference between the contents

of ergosterol in wild-type strain PdKH8 and the *PdMit1* deletion mutant was significant according to one-way ANOVA ($P < 0.05$). As shown in Fig. 6B, the ergosterol content was partially restored by introducing *PdMit1* into the *PdMit1* deletion mutant.

3.9. Disruption of *PdMit1* does not affect virulence

Virulence assays showed that disease symptoms on mature Ponkan fruits inoculated with the *PdMit1* deletion mutant were the same as those caused by wild-type strain PdKH8. Water-soaked lesions occurred on both strain-inoculated spots at 36 h after inoculation. At 4 dpi, the average diameter of maceration lesions caused by wild-type strain PdKH8 was 36.7 ± 1.0 mm, while that caused by the *PdMit1* deletion mutant was 31.9 ± 1.5 mm (Fig S4). Statistical analysis showed that their virulence was not significantly different ($P > 5\%$). These results suggest that disruption of *PdMit1* does not interfere with the virulence of *P. digitatum* on citrus fruit.

4. Discussion

In *S. cerevisiae*, there are two distinct MIPC synthase complexes, each of which consists of a catalytic subunit (Csg1p or Csh1p) and a shared regulatory subunit (Csg2p). *A. fumigatus* expresses a single MIPC synthase complex with the product of the *MitA* gene as the catalytic subunit [28]. Sequence analysis revealed that, like *MitA*, *PdMit1* is the only *P. digitatum* gene that is homologous to *Csg1*, *Csh1* and *MitA*, indicating that *P. digitatum* may express only a single MIPC synthase complex with the product of *PdMit1* as the catalytic subunit. Our results reported in this study suggest that *PdMit1* is important for hyphal growth, sporulation, conidial germination and calcium ion intolerance, but not pathogenicity.

A previous study demonstrated that *MitA* is essential for synthesis of MIPC and MIPC-derived complex glycosphingolipids in *A. fumigatus*, because the deletion of *MitA* renders MIPC undetectable in this fungus [18]. Based on the fact that *PdMit1* is the only MIPC synthase catalytic subunit that is highly homologous to *MitA*, we postulated that MIPC and its derivatives are also absent in the *PdMit1* deletion mutant. Therefore, *PdMit1* may regulate the aforementioned biological responses, likely through MIPC and MIPC-derived complex sphingolipids.

Compared to the wild-type strain, the content of ergosterol in the *PdMit1* deletion mutant was significantly increased (Fig. 6). MIPC and other complex sphingolipids have been suggested to form lipid rafts with cholesterol and sterols in the eukaryotic plasma membrane [10]. The increase in ergosterol may result from perturbation of lipid rafts due to the absence of their key components. Lipid rafts have important functions, including cell signaling, polarity and endocytosis [29]. Some of the phenotypic changes in the *PdMit1* deletion mutant could be associated with an alteration in the structure of lipid rafts. However, how the changed lipid rafts influence the growth, sporulation and conidial germination of *P. digitatum* remains to be experimentally explored.

Due to the absence of MIPC synthase, MIPC was absent in both *Csg1* and *Csg2* deletion mutants of *S. cerevisiae* and the *Csg1/MitA* deletion mutants of *Schizosaccharomyces pombe* and *A. fumigatus*, IPC-C, the precursor of MIPC, was aberrantly increased [15,16,18].

Exogenous Cu^{2+} or overexpression of the intracellular copper pump *Ccc2*, which hydroxylates IPC-C to IPC-D, rescued the growth defect of these mutants on the medium with high Ca^{2+} concentrations [15,18]; this suggests that accumulation of IPC-C, rather than the absence of MIPC and its complex sphingolipid derivatives, may be responsible for the hypersensitivity of yeast and fungal mutants deficient in MIPC synthase, including the *PdMit1* deletion mutant, to high concentrations of Ca^{2+} . In this study, under the stress of high Ca^{2+} , the vacuole in the wild-type strain of *P. digitatum* was enlarged, indicating that excessive cellular Ca^{2+} may be transferred into the vacuole (Fig. 4). However, the vacuole of the *PdMit1* deletion mutant failed to enlarge under high Ca^{2+} conditions, suggesting that *PdMit1* and its relative product MIPC and/or MIPC derivatives are required for vacuoles to function as a regulator of cytosolic Ca^{2+} . It was also reported that MIPC-deficient *S. Pombe* exhibited defective vacuolar morphology [16]. For most fungi, three Ca^{2+} -ATPases are responsible for the regulation of cellular Ca^{2+} concentration so as to maintain it at submicromolar levels. *PMCI* encodes a vacuolar Ca^{2+} -ATPase involved in Ca^{2+} sequestration and Ca^{2+} transport into the vacuole [30]. *PMR1* also encodes a P-type Ca^{2+} -ATPase in the Golgi membrane and pumps Ca^{2+} from the cytoplasm into the Golgi compartments [31]. *PMCA* (a plasma membrane Ca^{2+} -ATPase) is involved in Ca^{2+} efflux through the plasma membrane to control the cytoplasmic Ca^{2+} level [32]. In this study, the expression levels of *PMCI*, *PMR1* and *PMCA* orthologs in the wild-type strain of *P. digitatum* were significantly upregulated by 250 mmol/l Ca^{2+} . However, the *PdMit1* deletion mutant failed to increase expression of *PMCI*, *PMR1* and *PMCA* in response to treatment with 250 mmol/l Ca^{2+} , suggesting that *PdMit1* deletion impairs the ability of *P. digitatum* to handle excessive cellular Ca^{2+} through Ca^{2+} -ATPases, thus rendering them sensitive to Ca^{2+} .

With the participation of MIPC-derived glycolipid phospholipomannan, *C. albicans* could make contact with host cells and diffuse into the cell wall. Thus, the MIPC-derived glycolipid phospholipomannan is regarded as an important TLR2-specific PAMP (pathogen-associated molecular pattern) [19]. Due to reduced β -mannosylation of phospholipomannan, the *Mit1* deletion mutant of *C. albicans* was less virulent during both the acute and chronic phases of systemic infection in mice [17]. However, *MitA* deletion does not affect the virulence of *A. fumigatus* [18]. In this study, the disruption of *PdMit1* did not interfere with the virulence of *P. digitatum* on citrus fruit. These results suggest that MIPC and MIPC-derived complex sphingolipids may not be required for the pathogenicity of filamentous fungi.

In conclusion, we, for the first time, demonstrate that MIPC synthase plays an important role in the growth of mycelium, sporulation and conidial germination in a plant pathogenic fungus, probably by synthesizing MIPC and more complex sphingolipids or by preventing accumulation of IPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.resmic.2015.02.001>.

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Fig. 1. Comparison of mycelial growth of the *PdMit1* deletion mutant and the wild-type strain of *P. digitatum*. Conidial suspensions ($5 \mu\text{l } 1.0 \times 10^6$ conidia/ml) of the *PdMit1* deletion mutant, the *CPPdMit1* mutant and wild-type strain PdKH8 were inoculated onto PDA and CYA media at 25°C for 4 days (upper panel). The data on mycelial growth was collected and compared using one-way ANOVA (lower panel). Columns represent the average of colony diameters and bars represent standard errors calculated from three independent experiments.

Fig. 2.

Comparison of sporulation and conidial germination of the *PdMit1* deletion mutant and wild-type strain of *P. digitatum*. A) Comparison of sporulation of the *PdMit1* deletion mutant and wild-type strain PdKH8. They were inoculated on PDA media at 25 °C for 4 days. Conidia were collected and counted in a spectrophotometer at 5 dpi. B) Comparison of conidial germination of the *PdMit1* deletion mutant and wild-type strain PdKH8. Germination percent was assessed under a microscope at 8, 12, 16, 20 and 24 h after the conidial suspension was incubated on a PDA plate.

Fig. 3. Deletion of *PdMit1* increases the sensitivity of *P. digitatum* to Ca^{2+} . The conidial suspensions ($5 \mu\text{l } 1.0 \times 10^6$ conidia/ml) of the *PdMit1* deletion mutant, *CPPdMit1* mutant and wild-type strain *PdKH8* were inoculated on PDA media with 0, 100, 200, 300 and 400 mmol/l CaCl_2 at 25°C for 4 days.

Fig. 4.

Vacuolar morphology of wild-type strain PdKH8 and the *PdMit1* deletion mutant treated with 250 mmol/l CaCl_2 . Conidial suspensions ($100 \mu\text{l } 1.0 \times 10^7$ conidia/ml) of the *PdMit1* deletion mutant and wild-type strains of *P. digitatum* were added into PDB media supplemented with or without 250 mmol/l CaCl_2 , separately. The young mycelia were collected after inoculating for 24 h. The mycelia were then stained with neutral red for 10 min. Vacuolar morphology was observed under a microscope. Bars, $50 \mu\text{m}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Expression of Ca²⁺-ATPase in the *PdMit1* deletion mutant and wild-type strain of *P. digitatum* under Ca²⁺ stress. Mycelia were grown in PDB media for 36 h and then treated with 250 mmol/l CaCl₂ for 6 h. The relative expression levels of *PMCI*, *PMR1* and *PMCA* were measured on an Applied Biosystems 7300 real-time PCR system. The β-actin gene was used as an internal control. Columns represent the average of fold-changes. Bars represent the standard deviation.

Fig. 6. Deletion of *PdMit1* increased the sensitivity of *P. digitatum* to amphotericin B. A) Conidial suspensions of the *PdMit1* deletion mutant, the *CPPdMit1* mutant and wild-type strain PdKH8 were inoculated onto PDA containing 1 and 2 µg/ml of amphotericin B for 4 d. B) Analysis of the ergosterol contents of the *PdMit1* deletion mutant and wild-type strain PdKH8. The ergosterol was extracted from mycelia and analyzed by HPLC.