Wang et al. Phytopathology Research (2019) 1:16
https://doi.org/10.1186/s42483-019-0023-9

Phytopathology Research

RESEARCH Open Access

Phosphopholipid homeostasis plays an important role in fungal development, fungicide resistance and virulence in *Fusarium graminearum*

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Abstract

Phospholipids are major structural components of all cell membranes and participate in energy storage, signal transduction and environmental adaptability in eukaryotes. To date, the enzymes involved in phospholipid biosynthesis have been well characterized in budding yeast. However, their functions in filamentous fungi are largely unclear, especially their contribution to the interaction between phytopathogenic filamentous fungi and plants. In this study, we identified 10 phospholipid biosynthesis-related genes and genetically analyzed their functions in the Fusarium head blight pathogen *Fusarium graminearum*. The results of this study indicate that phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are critical for fungal vegetative growth. The biosynthesis of PE and PC is largely dependent on FgPsd2, FgCho2 and FgOpi3 in the de novo pathway of phospholipid biosynthesis in *F. graminearum*. Phospholipid biosynthetic gene mutants showed abnormal conidiation, increased sensitivity to fungicides and the oxidative stress agent H_2O_2, and defective endocytosis, especially the Δ*Fgpsd2*, Δ*Fgcho2* and Δ*Fgopi3* mutants. Importantly, this study shows for the first time that the de novo pathway of phospholipid biosynthesis is required for mycotoxin production and full virulence in plant pathogenic fungi.

Keywords: *Fusarium graminearum*, Fungicide resistance, Pathogenicity, The de novo pathway of phospholipid biosynthesis

Background

A phospholipid bilayer with embedded, integral and peripheral proteins constitutes the basic skeleton of the cell membrane, which acts as a selectively permeable barrier to isolate the cell from the external environment and ensure the independence of biochemical reactions in living organisms. Phospholipids can be further divided into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) based on their head groups (Nagle and Tristram-Nagle 2000). In eukaryotes and some prokaryotes, PE and PC are the most abundant phospholipids, accounting for more than 50% of the total phospholipid population (Vance and Steenbergen 2005).

Routes of PE and PC biosynthesis have been thoroughly investigated in the baker's yeast *Saccharomyces cerevisiae*. In yeast, PE and PC are synthesized via both endogenous (the de novo pathway) and exogenous pathways (the Kennedy pathway) (Daum et al. 1998; Gibellini and Smith 2010; Cassilly and Reynolds 2018). PS is synthesized from cytidyldiphosphate-diacylglycerol (CDP-DAG) and serine by the enzyme Cho1 with the associated release of cytidylmonophosphate (CMP) (Letts et al. 1983). Subsequently, PS is decarboxylated by phosphatidylserine decarboxylase 1 (Psd1p) localized in the inner mitochondrial membrane, and Golgi-localized phosphatidylserine decarboxylase 2 (Psd2p) to generate PE (Zinser et al. 1991; Trotter and Voelker 1995). Then, PE is methylated by two types of

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phospholipid methyltransferases, Cho2 and Opi3, using S-adenosyl-L-methionine as the methyl donor to yield PC (Daum et al. 1998; Gibellini and Smith 2010).

However, the Kennedy pathway incorporates exogenous ethanolamine (Etn) or choline (Cho) directly into PE or PC, respectively. Exogenous Etn and Cho are phosphorylated by the kinases Eki1 and Cki to generate phosphoethanolamine (EtnP) and phosphocholine (ChoP). EtnP and ChoP are subsequently catalyzed by the cytidylyltransferases Ect1 and Pct1, respectively, to generate cytidine diphosphate ethanolamine (CDP-Etn) and cytidine diphosphocholine (CDP-Cho). Finally, CDP-Etn and CDP-Cho react with DAG to form PE and PC, respectively (Birner et al. 2001). Mammals share conserved phospholipid biosynthesis pathways with yeast, but a major difference is found in PS biosynthesis (Kent 1995). In mammals, two phosphatidylserine synthases, Pss1 and Pss2, exchange serine for Etn in PE or for Cho in PC to generate PS, unlike the Cho1-dependent process of PS synthesis in yeast (Sturbois-Balcerzak et al. 2001; Bergo et al. 2002). A genomic survey indicates that Cho1, which has been suggested as a potential antifungal drug target, is conserved in fungi but absent in mammals (Braun et al. 2005).

In addition to their role in the formation of membrane bilayers, phospholipid biosynthetic genes also directly or indirectly participate in cytokinesis, cell wall integrity, secretion and several other biological processes (Wolf et al. 2015). For instance, PS synthase is required for the proper regulation of cellular morphogenesis and cytokinesis in *Schizosaccharomyces pombe*. Deletion of the PS synthase gene caused cell necrosis and severe morphological and cytokinesis abnormalities, including bent, bulbous, branched, and/or ovoid cells (Matsuo et al. 2007). The *Candida albicans Δ/Δcho1* mutant exhibits defects in cell wall integrity and mitochondrial function (Chen et al. 2010). The *Δ/Δcho1* and *Δ/Δpsd1Δ/Δpsd2* strains showed defects in extracellular vesicle morphology and cargo, and immunostimulatory properties (Wolf et al. 2015). Moreover, accumulating evidence suggests that phospholipids are also important for the interaction of symbiotic and pathogenic microorganisms with host cells. The PC level in *Bradyrhizobium japonicum* membranes is critical for efficient symbiosis with the soybean host plant (Minder et al. 2001). Disruption of the PS synthase Pssa impaired several virulence traits in *Brucella abortus*, such as intracellular survival in macrophages and HeLa cells, maturation of the replicative Brucella-containing vacuole, and mouse colonization (Bukata et al. 2008). *Agrobacterium tumefaciens* mutants unable to synthesize PC showed a defective type IV secretion system and impaired tumor formation on Kalanchoë plants (Wessel et al. 2006). Both PS synthesis and de novo PE synthesis are required for *C. albicans* virulence (Chen et al. 2010). However, little is known about the functions of phospholipids in filamentous fungi, especially their contribution to the interaction between pathogenic filamentous fungi and host cells.

The filamentous fungus *Fusarium graminearum* is the predominant pathogenic agent of the devastating Fusarium head blight (FHB) disease of wheat (Xu and Nicholson 2009). Epidemics and the incidence of FHB directly cause severe yield losses in FHB-prone regions worldwide. For example, an FHB epidemic with a high incidence rate has resulted in the infection of approximately 20% of the planting area of wheat since 2010 in China (Chen et al. 2017). More critically, *F. graminearum* produces harmful mycotoxins, such as deoxynivalenol (DON), nivalenol and zearalenone, in infested grains (Tang et al. 2018a). Currently, the application of chemical fungicides remains the main approach to control FHB due to the unavailability of FHB-resistant wheat cultivars (Blandino et al. 2012). Unfortunately, fungicide-resistant *F. graminearum* strains have been detected in the field after intensive applications of fungicides (Zhang et al. 2013). Moreover, the application of several fungicides, including carbendazim and azoles, at sublethal concentrations triggers DON biosynthesis (Milus and Parsons 1994; Simpson et al. 2001; Magan et al. 2002; Zhang et al. 2009; Audenaert et al. 2010; Tang et al. 2018a). Therefore, the identification of potential drug targets for the development of new fungicides is imperative.

In this study, we identified phospholipid biosynthetic genes and genetically analyzed their functions in *F. graminearum*. The results of this study indicate that PE and PC are critical for mycelial growth and that the biosynthesis of PE and PC is largely dependent on the de novo pathway in *F. graminearum*. Importantly, this study shows for the first time that the de novo pathway of phospholipid synthesis participates in the mycotoxin production and full virulence of this important phytopathogenic fungus.

Results

Identification of genes participating in PE and PC biosynthesis

The sequences of all enzymes directly involved in the PE and PC biosynthetic pathway in budding yeast were used as queries for BlastP searches to identify homologous genes in the genome of *F. graminearum*. A total of 10 proteins were identified, and their functions in *F. graminearum* were predicted (Table 1). Next, these proteins were assigned to the locations of their yeast counterparts (Fig. 1a) based on the biosynthetic pathway of PE and PC in yeast. Although the identified proteins involved in PE and PC biosynthesis in *F. graminearum* shared relatively low identity (31%–54%) with their homologs (Table 1), structural analysis revealed that these proteins contained the same functional

Table 1 Predicted proteins involved in the PE and PC biosynthesis pathway in *F. graminearum*

Protein in yeast	Function	Homologues in *F.graminearum*	Identities (%)	E-value
Cho1	CDP-diacylglycerol serine O-phosphatidyltransferase	FgCho1 (FGSG_06370)	54	3.6e-61
Psd1	Phosphatidylserine decarboxylase in the mitochondrion	FgPsd1(FGSG_01865)	46	4.1e-92
Psd2	Phosphatidylserine decarboxylase 2	FgPsd2 (FGSG_10007)	50	1.6e-102
Cho2	Phosphatidyl-ethanolamine methyltransferase	FgCho2 (FGSG_05066)	32	5.3e-104
Opi3	Methylene-fatty-acyl-phospholipid synthase	FgOpi3 (FGSG_08613)	51	1.0e-55
Eki1/Cki1	Ethanolamine kinase/ choline kinase	FgCki1 (FGSG_17231)	31	3.4e-38
Ect1	Ethanolamine-phosphate cytidylyltransferase	FgEct1 (FGSG_04571)	32	2.8e-43
Ept1	Ethanolamine phosphotransferase	FgEpt1 (FGSG_08706)	37	6.2e-67
Pct1	Choline-phosphate cytidylyltransferase	FgPct1(FGSG_10005)	54	4.5e-81
Cpt1	Cholinephosphotransferase	FgCpt1 (FGSG_09402)	35	1.3e-48

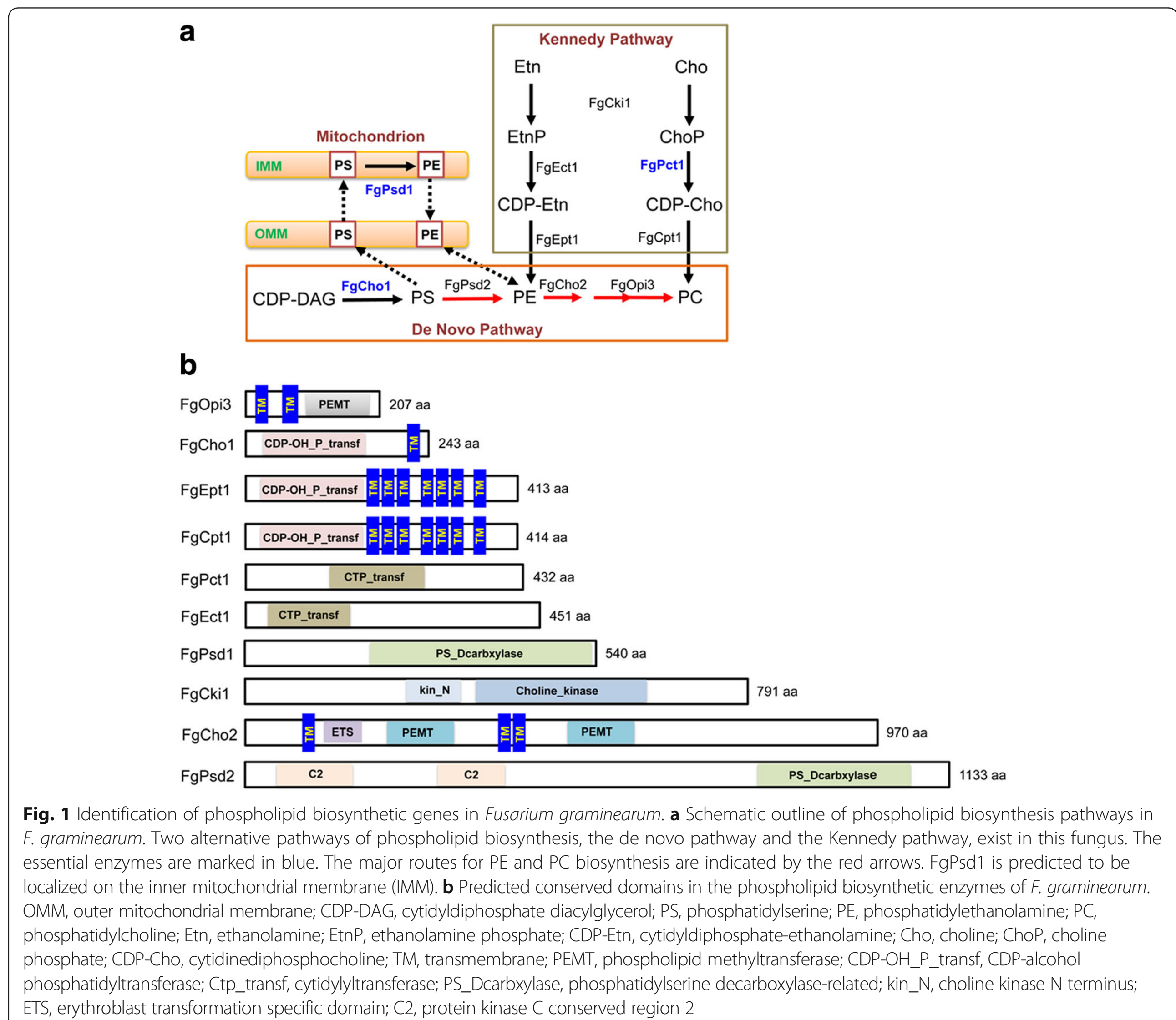

Fig. 1 Identification of phospholipid biosynthetic genes in *Fusarium graminearum*. **a** Schematic outline of phospholipid biosynthesis pathways in *F. graminearum*. Two alternative pathways of phospholipid biosynthesis, the de novo pathway and the Kennedy pathway, exist in this fungus. The essential enzymes are marked in blue. The major routes for PE and PC biosynthesis are indicated by the red arrows. FgPsd1 is predicted to be localized on the inner mitochondrial membrane (IMM). **b** Predicted conserved domains in the phospholipid biosynthetic enzymes of *F. graminearum*. OMM, outer mitochondrial membrane; CDP-DAG, cytidyldiphosphate diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Etn, ethanolamine; EtnP, ethanolamine phosphate; CDP-Etn, cytidyldiphosphate-ethanolamine; Cho, choline; ChoP, choline phosphate; CDP-Cho, cytidinediphosphocholine; TM, transmembrane; PEMT, phospholipid methyltransferase; CDP-OH_P_transf, CDP-alcohol phosphatidyltransferase; Ctp_transf, cytidylyltransferase; PS_Dcarbxylase, phosphatidylserine decarboxylase-related; kin_N, choline kinase N terminus; ETS, erythroblast transformation specific domain; C2, protein kinase C conserved region 2

domains as their yeast counterparts (Fig. 1b). The kinase Eki1 and its paralog Cki1 phosphorylate ethanolamine and choline, respectively, in the yeast Kennedy pathway. However, *F. graminearum* FGSG_17231 is homologous to both yeast Eki1 and Cki1, which indicates that the protein encoded by the FGSG_17231 locus may have both ethanolamine kinase and choline kinase activity; this protein is hereafter designated FgCki1. Taken together, the data from the bioinformatic analysis suggest that proteins participating in PE and PC biosynthesis are relatively conserved in yeast and filamentous fungi, at least in *F. graminearum*.

Biosynthesis of PE and PC is largely dependent on the de novo pathway

To characterize the function of the above 10 identified PE and PC biosynthetic genes, we generated deletion mutants of each gene using the homologous recombination approach. After the screening step, hygromycin-resistant transformants were identified as successful deletion mutants by polymerase chain reaction (PCR) amplification with the primer pair P5 + P6 (Additional file 1: Table S1) and further analyzed via a Southern blot assay (Additional file 2: Figure S1). Among these mutants, 7 genes—*FgPSD2*, *FgCHO2*, *FgOPI3*, *FgECT1*, *FgEPT1*, *FgCKI1* and *FgCPT1*—were successfully deleted individually. For *FgCHO1*, *FgPSD1* and *FgPCT1*, we failed to identify true gene knockout mutants after screening over 100 ectopic transformants from at least three independent transformation experiments, indicating that the deletion of these genes may be lethal because of the high homologous recombination efficiency in *F. graminearum* (Yun et al. 2015).

All resulting gene deletion mutants were characterized for defects in mycelial growth on various media. The phenotypes of at least three knockout mutants of each gene were examined. As shown in Fig. 2 and Additional file 3: Figure S2, the hyphal growth rate of each mutant was not significantly different from that of the wild-type PH-1 strain on complete medium (CM), except for *ΔFgect1* and *ΔFgcki1*, which exhibited a 15% reduction in growth. Mutants in which the single circuitry of the Kennedy pathway was disrupted—Etn-EtnP-CDPEtn-PE (*ΔFgect1* and *ΔFgept1*) or Cho-ChoP-CDPCho-PC

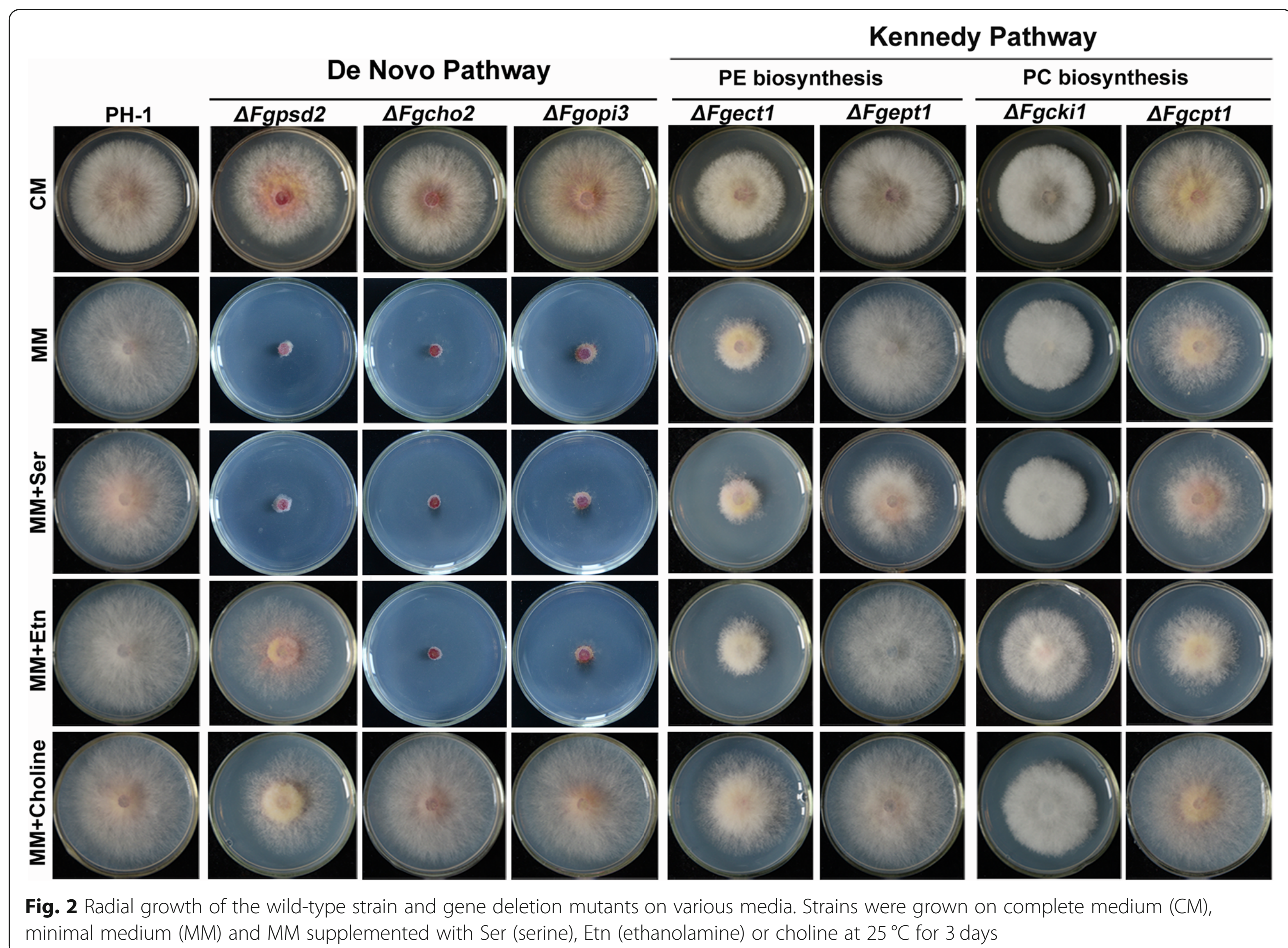

Fig. 2 Radial growth of the wild-type strain and gene deletion mutants on various media. Strains were grown on complete medium (CM), minimal medium (MM) and MM supplemented with Ser (serine), Etn (ethanolamine) or choline at 25 °C for 3 days

(*ΔFgcki1* and *ΔFgcpt1*)—were able to grow on minimal medium (MM). However, all three deletions of genes in the de novo pathway of phospholipid biosynthesis (the *ΔFgpsd2*, *ΔFgcho2* and *ΔFgopi3* mutants) almost abolished vegetative growth on MM.

To test whether the growth phenotypes of the mutants were caused by defects in PE or PC, MM was supplemented individually with the initial precursors in different subpathways—serine, ethanolamine and choline—to rescue the defects. Clearly, supplementation with serine was not able to rescue the growth defects of the mutants. This result suggested that FgPsd1 played a minor role in PE biosynthesis and could not serve as an alternative PE source to support mycelial growth in the absence of *FgPSD2*. In addition, the growth defects of *ΔFgpsd2* but not *ΔFgcho2* and *ΔFgopi3* were partially compensated by the addition of exogenous ethanolamine, which was presumed to increase the amount of cellular PE through the Etn-EtnP-CDPEtn-PE process in the Kennedy pathway (Fig. 1a). Notably, the increased amount of PC generated by supplementation with choline rescued the growth defects in all mutants to different extents. Taken together, these findings indicate that the biosynthesis of PE and PC largely depends on the de novo pathway. Moreover, the de novo pathway is critical for hyphal growth in *F. graminearum*.

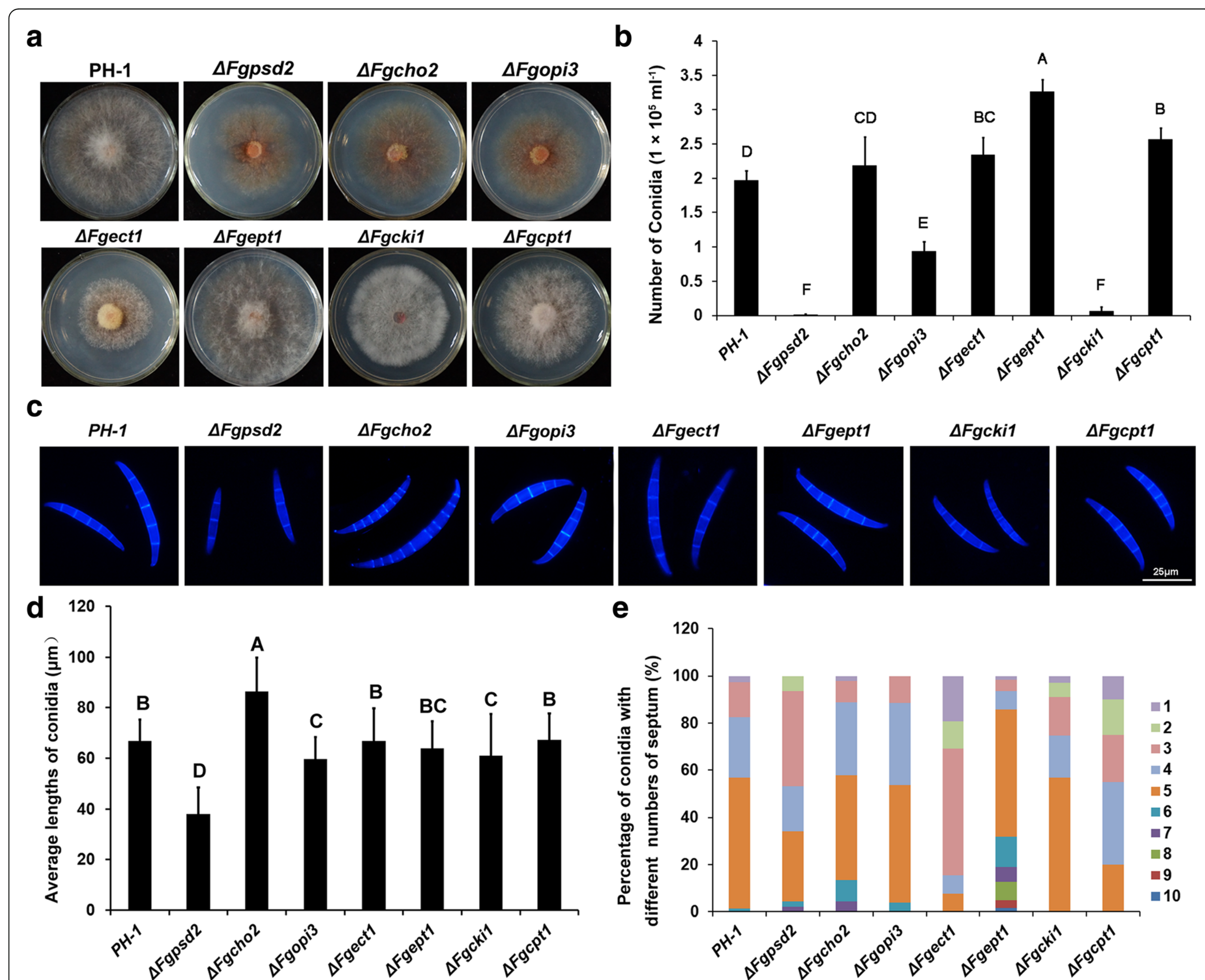

Fig. 3 Phospholipid biosynthetic genes are involved in conidiation. **a** Radial growth of the wild-type strain and deletion mutants on the conidiation-inducing medium CMC. **b** The number of conidia of each strain after 4 days of incubation in 100 mL of CMC medium. **c** Conidial morphology of the wild-type and deletion mutants. Septa were stained with calcofluor white and observed by fluorescence microscopy. Bar = 25 μm. **d** Conidial length of the wild-type and deletion mutants. **e** Comparisons of the ratio of the different numbers of septa in conidia of the wild-type strain and deletion mutants harvested from 4-day-old CMC cultures. The vertical lines on the bars indicate the standard deviations of three repeated experiments. The values corresponding to the bars denoted with the same letter are not significantly different according to the least significant difference (LSD) test at $P = 0.01$

PE and PC biosynthetic genes are involved in conidiation

To determine the role of PE and PC biosynthetic genes in asexual reproduction, the number of conidia generated by the individually tested strains in carboxymethyl cellulose (CMC) medium was calculated. All strains were able to grow on the CMC agar plates (Fig. 3a). After 4 days of incubation, the wild-type strain produced approximately 2×10^5 conidia/mL, while the *ΔFgpsd2, ΔFgopi3* and *ΔFgcki1* strains produced significantly fewer conidia than the wild-type strain. In contrast, the *ΔFgept1* strain produced 3.3×10^5 conidia/mL, significantly more than the wild-type strain (Fig. 3b). To further examine the conidial morphology, conidia were stained with calcofluor white and observed under a fluorescence microscope. As shown in Fig. 3c-e, the average size of conidia produced by the *ΔFgpsd2* strain was 38 ± 9.4 μm, which was smaller than that of the wild-type strain (67 ± 8.7 μm). Moreover, conidia of the *ΔFgpsd2* strain exhibited fewer septa than conidia of the wild-type strain. Most of the conidia (approximately 54%) produced by the mutant strains had three or fewer septa, whereas the majority of conidia produced by the wild-type strain had five septa (Fig. 3b-d). Interestingly, the *ΔFgcho2* deletion mutant produced larger conidia (86 ± 7.8 μm) with more septa than the wild-type strain. Over 30% of these conidia contained more than five septa, or even ten septa. In the complemented strains *ΔFgpsd2-C*, *ΔFgcho2-C* and *ΔFgopi3-C*, the conidia production and morphological defects of the corresponding mutants were rescued (Additional file 4: Figure S3a-c).

Mutants of the de novo pathway show increased sensitivity to multiple antifungal agents

In previous studies, it has been reported that membrane lipids are involved in drug susceptibility, cold stress and other abiotic stresses (Welti et al. 2002; Gulshan et al. 2010; Khandelwal et al. 2018). Therefore, we were interested in determining the susceptibility of the *F. graminearum* mutants to various stresses, including fungicide treatment, osmotic stress and cell wall stress. As shown in Fig. 4a and b, the *ΔFgpsd2*, *ΔFgcho2* and *ΔFgopi3* mutants exhibited significantly increased sensitivity to rapamycin, carbendazim, phenamacril and tebuconazole. Among the mutants, the *ΔFgpsd2* mutant exhibited the highest sensitivity to the four tested fungicides. However, the *ΔFgpsd2* mutant grew similar to the wild-type strain in medium supplemented with osmotic or cell wall stress agents (data not shown). Reintroduction of the wild-type allele restored the sensitivity of all three mutants towards the tested fungicides to that of the PH-1 strain (Additional file 4: Figure S3d, e).

The de novo pathway of phospholipid biosynthesis is required for full virulence

The virulence of each mutant was evaluated by point inoculation of a conidial suspension on flowering wheat heads. On wheat heads inoculated with the wild-type strain PH-1, scab symptoms first developed on the inoculated spikelets and rapidly spread to the neighboring spikelets, causing serious and typical Fusarium head blight symptoms after two weeks of inoculation. However, under the same conditions, the mutants of the de

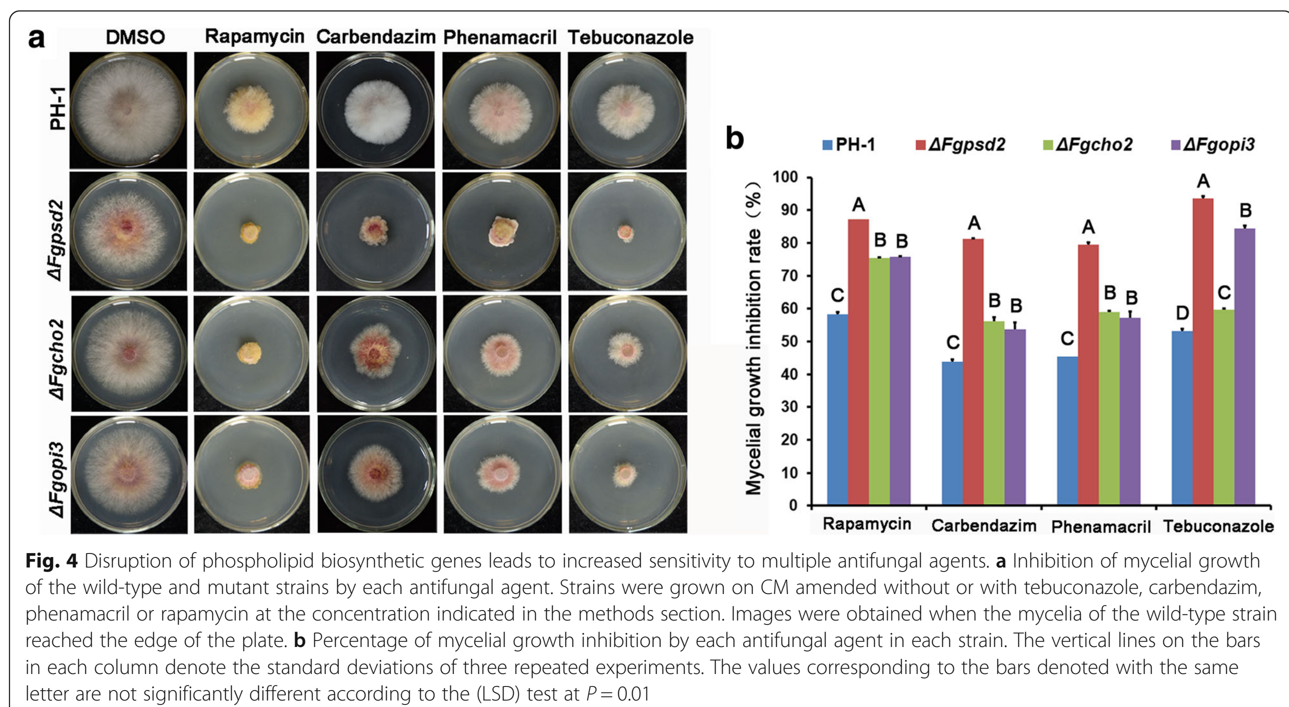

Fig. 4 Disruption of phospholipid biosynthetic genes leads to increased sensitivity to multiple antifungal agents. **a** Inhibition of mycelial growth of the wild-type and mutant strains by each antifungal agent. Strains were grown on CM amended without or with tebuconazole, carbendazim, phenamacril or rapamycin at the concentration indicated in the methods section. Images were obtained when the mycelia of the wild-type strain reached the edge of the plate. **b** Percentage of mycelial growth inhibition by each antifungal agent in each strain. The vertical lines on the bars in each column denote the standard deviations of three repeated experiments. The values corresponding to the bars denoted with the same letter are not significantly different according to the (LSD) test at $P = 0.01$

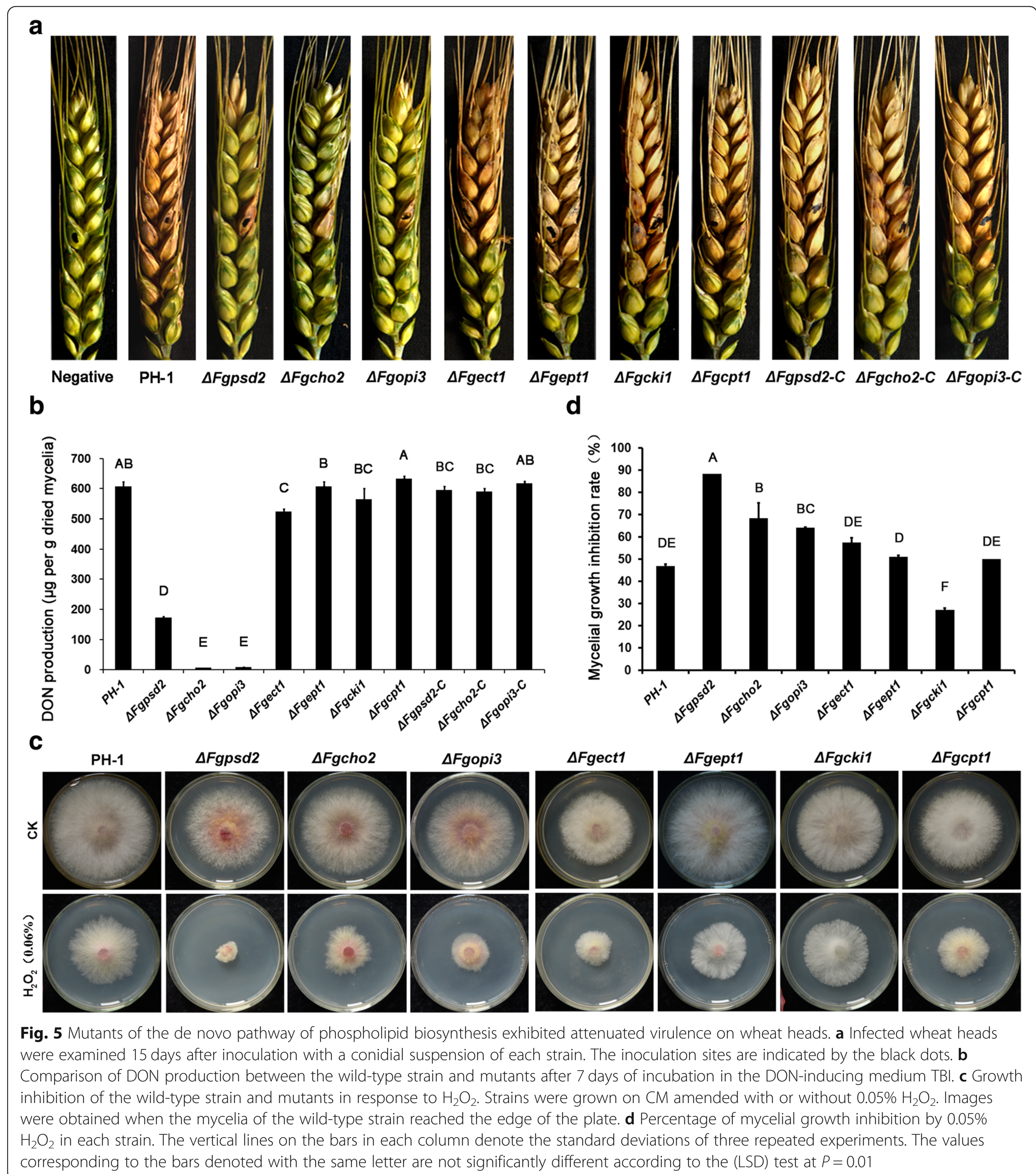

Fig. 5 Mutants of the de novo pathway of phospholipid biosynthesis exhibited attenuated virulence on wheat heads. **a** Infected wheat heads were examined 15 days after inoculation with a conidial suspension of each strain. The inoculation sites are indicated by the black dots. **b** Comparison of DON production between the wild-type strain and mutants after 7 days of incubation in the DON-inducing medium TBI. **c** Growth inhibition of the wild-type strain and mutants in response to H_2O_2. Strains were grown on CM amended with or without 0.05% H_2O_2. Images were obtained when the mycelia of the wild-type strain reached the edge of the plate. **d** Percentage of mycelial growth inhibition by 0.05% H_2O_2 in each strain. The vertical lines on the bars in each column denote the standard deviations of three repeated experiments. The values corresponding to the bars denoted with the same letter are not significantly different according to the (LSD) test at $P = 0.01$

novo pathway (*ΔFgpsd2, ΔFgcho2* and *ΔFgopi3*) failed to spread from the inoculated floret to the rachis and caused scab symptoms only in the inoculated spikelets (Fig. 5a).

DON is an important virulence factor and plays critical roles in the spread of the fungus within host tissues (Proctor et al. 1995; Desjardins et al. 1996). Since the deletion mutants of the de novo pathway attenuated disease spread *in planta*, DON production was examined. As expected, these three mutants produced significantly less DON than the wild-type strain after 7 days of incubation in trichothecene biosynthesis induction (TBI) medium (Fig. 5b). In addition, these three mutants were

more sensitive than the wild-type strain to H_2O_2, which is a common oxidative agent generated by host plants during fungal infection. Complemented strains of these three mutants and other mutants in the Kennedy pathway produced similar amounts of DON and caused the same disease severity on wheat heads (Fig. 5). Collectively, these findings indicate that the de novo pathway of phospholipid biosynthesis plays an important role in the full virulence of *F. graminearum*.

Disruption of phospholipid synthesis impairs fungal endocytosis

The phospholipid composition of cellular membranes has been suggested to be required for the intracellular trafficking of some transporters (Guo et al. 2010; Lee et al. 2012). The endocytosis of mutants was therefore examined using a FM4–64 staining assay. The plasma membrane and septa were quickly stained by FM4–64 within 1 min in both the wild-type strain and the mutants. FM4–64 dye was taken up, and a fluorescence signal clearly appeared on the plasma-facing membrane of intracellular organelles, such as vacuoles and endosomes, after 5 min of staining in the mycelia of the wild-type strain. However, the uptake of the fluorescent dye was appreciably impaired in all mutants tested, even after 10 min of incubation (Fig. 6). These results suggest that the biosynthesis of PS, PE and PC is important for normal endocytosis.

Discussion

Lipids are important biomolecules and include phospholipids, fatty acids, sterols, and other molecules. The functions of lipids include storing energy, signaling, and acting as structural components of cell membranes (Subramaniam et al. 2011). Cells use ~ 5% of their genes to synthesize lipids for cell viability, suggesting the complexity and importance of lipids (Sud et al. 2007; Van Meer et al. 2008). Because of this complexity and the differences in lipid biosynthetic and metabolic pathways between fungal and mammalian cells, the key enzymes in these pathways have been suggested to be targets of fungicides (Pan et al. 2018). For instance, azoles, polyene macrolides and other inhibitors have been developed to interfere with ergosterol biosynthesis to suppress fungal infection (Ghannoum and Rice 1999). Phospholipids are critical structural components of cell membranes and are important for fungal growth. In *C. albicans*, interruption of the de novo pathway of phospholipid biosynthesis by knocking out *CHO1* or double mutation of *PSD1/PSD2* caused defects in cell wall integrity, mitochondrial function, filamentous growth and virulence (Chen et al. 2010; Wolf et al. 2015; Cassilly and Reynolds 2018). The *S. cerevisiae Δpsd1* deletion mutant with a blockade of the major PE synthesis pathway was unable to be cultivated on lactate or ethanol without supplementation with Etn, Cho or Ser (Birner et al. 2001). In the filamentous fungi *Aspergillus nidulans* and *Pestalotiopsis microspora*, the loss of ChoC (Opi3 in yeast) resulted in defects in PC production, vegetative growth and

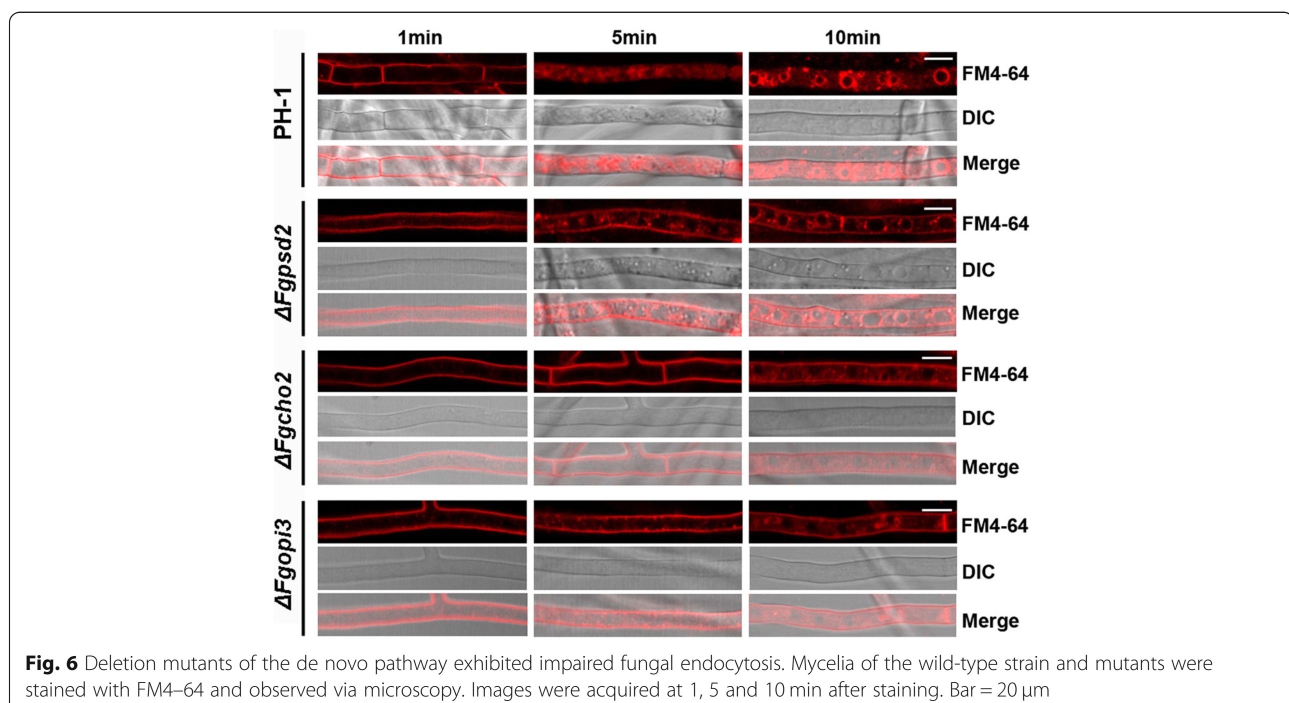

Fig. 6 Deletion mutants of the de novo pathway exhibited impaired fungal endocytosis. Mycelia of the wild-type strain and mutants were stained with FM4–64 and observed via microscopy. Images were acquired at 1, 5 and 10 min after staining. Bar = 20 μm

conidiation (Tao et al. 2010; Akhberdi et al. 2018). More importantly, Cho1 and Opi3 (ChoC) do not have homologs in mammalian cells. Therefore, new drugs targeting Cho1 and Opi3 could potentially be developed for antifungal therapy or fungal disease control (Pan et al. 2018). In agreement with this concept, 7-chloro-N-(4-propylphenyl)-4-quinolinamine (7CPQA) has been shown to reduce *Plasmodium yoelii* infection in mice, with low toxicity towards mammalian cells, via the inhibition of PSD activity.

In this study, 10 yeast homologous genes involved in PS, PE and PC biosynthesis were identified in *F. graminearum* (Fig. 1 and Table 1). Genetic evidence indicates that the de novo pathway is the major route for PS, PE and PC biosynthesis in this fungus. Consistent with the results of previous studies, the de novo pathway was essential for cell growth in minimal medium and participated in mediating asexual development and virulence in *F. graminearum* (Fig. 2, Fig. 3 and Fig. 5). These findings imply that a new antifungal drug targeting a key enzyme in the de novo pathway may be used for FHB disease management in the future. Our results also provide additional evidence to support the hypothesis that the inhibition of Cho1 or Opi3 (ChoC) activity is likely to have a broad-spectrum antifungal effect.

In *S. cerevisiae*, Psd1 is a major catalytic enzyme of PE formation, which accounts for approximately 80% of PSD activity in vivo. Loss of Psd2p activity does not affect cell growth (Burgermeister et al. 2004). In contrast, the *ΔFgpsd2* deletion mutant of *F. graminearum* was not able to grow on minimal medium without supplementation with Etn or Cho (Fig. 2), indicating that the PE deficiency in the *ΔFgpsd2* mutant cannot be rescued by the activity of FgPsd1 and that FgPsd2 is the major enzyme of PC and PE formation in *F. graminearum*. In addition, we failed to generate the *FgPSD1, FgCHO1* and *FgPCT1* gene mutants even with high homologous recombination efficiency in *F. graminearum*, indicating that these genes may be essential for growth and have biological functions in addition to phospholipid synthesis. However, their yeast counterparts are not essential. Collectively, the evidence presented here suggests that partial variation in the metabolic network of phospholipids exists between yeasts and filamentous fungi.

The close link between phospholipid homeostasis and drug resistance has been demonstrated. Overproduction of Psd1 induced the transcription of the gene encoding the ATP-binding cassette transporter Pdr5 and increased cycloheximide resistance in yeast (Gulshan et al. 2008). The *C. albicans* phospholipid mutants *Δ/Δ*cho1 and *Δ/Δpsd1/2* showed increased sensitivity to farnesol (Hasim et al. 2018). Recently, Khandelwal et al. (2018) reported that the *Δ/Δpsd1Δ/Δpsd2* mutant showed increased membrane fluidity and a reduced plasma membrane dipole potential, which subsequently resulted in increased drug accumulation and azole susceptibility in *C. albicans*. Similarly, dysfunction of phospholipid homeostasis in *F. graminearum* induced by disrupting biosynthetic genes, especially *FgPSD2,* also resulted in increased sensitivity to multiple tested fungicides (Fig. 4). Further studies will analyze membrane fluidity and the transcription of multidrug transporters in *ΔFgpsd2* to reveal the mechanism of multidrug sensitivity. Therefore, this line of evidence indicates that altering phospholipid homeostasis is likely to be an alternative approach to manage multidrug resistance problems in clinical and agricultural fungal infections and diseases.

Phospholipid biosynthesis is also important for virulence in pathogenic fungi. The concentration of PC was higher in virulent strains than in an avirulent strain of *Paracoccidioides brasiliensis* (Vallejo et al. 2012). Defects in phospholipid biosynthesis resulted in attenuated virulence in *C. albicans* and the insect pathogenic fungus *Metarhizium robertsii* (Chen et al. 2010; Gao et al. 2016; Chen et al. 2018b). Here, we found that the major phospholipid biosynthetic gene mutants, including *ΔFgpsd2, ΔFgcho2* and *ΔFgopi3,* exhibited dramatically attenuated virulence *in planta* (Fig. 5a). The markedly reduced invasive growth of the mutants was caused by defects in multiple virulence factors. All three mutants exhibited significantly reduced production of DON (Fig. 5b), which has been identified as a critical virulence factor and plays a significant role in the spread of the fungus within wheat spikes (Proctor et al. 1995; Desjardins et al. 1996; Seong et al. 2009). Moreover, disruption of the de novo pathway led to increased sensitivity of *F. graminearum* to oxidative stress. It is well known that the earliest defense event in response to fungal infection, including in the Fusarium–host interaction, is the production of reactive oxygen species (ROS), such as H_2O_2, by the plant cell (Lehmann et al. 2015). The results of our previous studies also demonstrated that tolerance to H_2O_2 is important for *F. graminearum* pathogenicity (Chen et al., 2018a; Tang et al., 2018b). In addition, the reduction in the growth of the mutants may be responsible for their attenuated virulence. In conclusion, defects in growth, ROS tolerance and DON production contribute to the attenuated virulence of *F. graminearum* mutants of the de novo pathway to host plants.

Conclusions

The results of this study indicate that PE and PC biosynthesis are largely dependent on the de novo pathway of phospholipid biosynthesis in *F. graminearum.* Phospholipid biosynthetic genes, especially genes in the de novo pathway of phospholipid biosynthesis, are required for fungal development, abiotic stress adaptation, mycotoxin production and full virulence in this plant pathogenic fungus.

Methods

Strains and culture conditions

The *F. graminearum* wild-type strain PH-1 was used as the parental strain for transformation experiments. The wild-type strain and the mutants generated in this study were grown on minimal medium (MM) [10 mM K_2HPO_4, 10 mM KH_2PO_4, 4 mM $(NH_4)_2SO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 9 mM $FeSO_4$, 10 mM glucose and 1 L water (pH 6.9)], complete medium (CM), or yeast extract peptone dextrose (YEPD) liquid medium [1%(g/v) peptone, 0.3% (g/v) yeast extract, 2%(g/v) glucose and 1 L water (pH 6.7)]; conidiation was assessed in carboxymethyl cellulose (CMC) liquid medium [0.1% (g/v) carboxymethyl cellulose, 10 mM KH_2PO_4, 4 mM NH_4NO_3, 2 mM $MgSO_4$ and 1 L water] as described previously (Liu X et al. 2013). For the induction of DON biosynthesis, strains were grown in liquid trichothecene biosynthesis induction (TBI) medium (30 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4·7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4·7H_2O$, 1.47 g putrescine hydrochloride, trace elements and 1 L water (pH 4.5)] at 25 °C in the dark (Gardiner et al. 2009).

Generation of gene deletion mutants

Gene deletion and complementation strains were generated using a protocol described previously (Yu et al. 2014). The primers used to amplify the flanking sequences for each gene are listed in Additional file 1: Table S1. The resulting PCR products were transformed into protoplasts of the wild-type strain PH-1, as described previously (Hou et al. 2002). Putative gene deletion transformants were selected on PDA medium supplemented with hygromycin (100 μg/mL), identified by PCR assays with the relevant primers (Additional file 1: Table S1) and further confirmed by Southern blot assays. To complement the *ΔFgpsd2* mutation, PCR products including the native promoter, open reading fragment of the gene and a geneticin resistance cassette were fused and transformed into the *ΔFgpsd2* mutant. Transformants were selected by geneticin (100 μg/mL), identified by PCR and designated *ΔFgpsd2-C*. The complementation strains *ΔFgcho2-C* and *ΔFgopi3-C* were constructed via a similar approach. All mutants generated in this study were preserved in 15% glycerol at -80 °C.

Fungicide sensitivity and stress response determination

To assess fungal growth under environmental stress conditions, mycelial plugs (5 mm in diameter) taken from the periphery of a 3-day-old colony of each strain were inoculated on CM amended with the following compounds: carbendazim (0.3 μg/mL), phenamacril (0.25 μg/mL), tebuconazole (0.25 μg/mL), or rapamycin (0.025 μg/mL). To determine the sensitivities of these mutants to osmotic or cell wall stress agents, fresh mycelia were inoculated on CM amended with the following products: Paraquat (100 μg/mL) and 0.05% H_2O_2, caffeine (0.75 μg/mL), SDS (10 μg/mL), Congo Red (0.3 μg/mL), or calcofluor white (CFW, 100 μg/mL). The growth rescue experiment was conducted on MM amended with 0.04% (v/v) ethanolamine (Etn), choline (5 mM) and serine (1 mM). After the plates were incubated at 25 °C for 3 days, images were obtained, and the colony diameters on each plate were measured. Each experiment was repeated independently three times.

Conidiation assay

The conidiation of each strain was induced in CMC liquid medium, and conidia were collected for later research. Briefly, fresh mycelia of each strain (50 mg) taken from the periphery of a 3-day-old colony were inoculated in a 250 mL flask containing 100 mL of CMC medium. The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm). For each strain, the number of conidia in the broth was determined using a hemacytometer. In addition, the strains were incubated on CMC agar plates. The conidial morphology and lengths and the number of septa were observed with a confocal microscope after CFW staining.

Virulence detection assay

Conidia of each strain were resuspended in sterile water to a concentration of 1×10^5 conidia/mL. A 10 μL aliquot of conidial suspension was injected into a floret in the central spikelet of a single flowering wheat head of the susceptible cultivar Jimai 22. There were ten replicates for each strain. After inoculation, the plants were maintained at 22 ± 2 °C under 95%–100% humidity. Fifteen days after inoculation, the number of infected spikelets in each inoculated wheat head was recorded. The experiment was repeated three times.

DON production assay

Fresh mycelia of each strain were induced in TBI liquid medium at 28 °C with agitation (180 rpm) and were then filtered with gauze on the seventh day after incubation. The DON content in each sample was extracted and measured by a Waters 1525 HPLC system (Palo Alto,CA, USA) as described previously (Liu et al. 2013). The experiment was repeated independently three times.

Confocal microscopy and image analysis

A 7.5 mM stock solution of FM4–64 (catalog no. T13320; 10 × 100 μg; Invitrogen) was prepared by dissolving 100 μg of FM4–64 in 108 μL of DMSO. A working solution of 7.5 μM was prepared by diluting 1 μL of the stock solution in 1 mL of water. Confocal microscopy was performed with a Zeiss LSM 780 Meta laser

scanning confocal microscope. FM4–64 was excited using a 515 nm laser and emission was observed at 640 nm. CFW was excited using a 395 nm laser and emission was observed at 440 nm. Images were processed using Zen software (Black edition, version 10.0, Zeiss).

Additional files

Additional file 1: Table S1. A list of PCR primers used in this study. (DOCX 18 kb)

Additional file 2: Figure S1. Schematic representation of gene disruption strategy and Southern blot analyses of the deletion mutants. **a** Schematic representation of the disruption strategy for *Fgpsd2*, *Fgcho2*, *Fgopi3*, *Fgect1*, *Fgcpt1*, *Fgept1* and *Fgcki1*. Black arrows indicate hygromycin resistance gene (*HPH*). **b** PCR assays for identification of the gene deletion mutants. **c** Southern blot analyses of the mutants. A fragment of *HPH* gene was used as the probe in the Southern blot assays. The restriction enzyme used for digestion of genomic DNA preparation was indicated in schematic representation of the disruption strategy for each strain. The size of the resulting hybridization band was indicated for each strain at the bottom of Southern blot images. (TIF 8725 kb)

Additional file 3: Figure S2. Relative mycelial growth rate of mutants on completed medium to that of the wild-type PH-1. Strains were grown on CM at 25 °C for 3 days. Line bars in each column denote standard deviations of three repeated experiments. Bars followed by the same letter are not significantly different according to LSD test at $P = 0.01$. (TIF 2364 kb)

Additional file 4: Figure S3. Phenotypes of complementation mutants in the de novo pathway. **a** Conidial morphology of the wild-type and complementation mutants. The septa were stained with calcofluor white and observed by fluorescent microscope. Bar = 25 μm. **b** The number of conidia of each strain after 4 days of incubation in 100 mL CMC medium. **c** Conidial length of the wild-type and complementation mutants. **d** Mycelial growth inhibition of the wild-type and mutants towards each antifungal agent. Strains were grown on the CM amended without or with tebuconazole, carbendazim, phenamacril or rapamycin at the concentration indicated in the material and methods. **e** Mycelial growth inhibition rate of mutants towards antifungal agents. Line bars in each column denote standard deviations of three repeated experiments. Bars followed by the same letter are not significantly different according to LSD test at $P = 0.01$. (TIF 10888 kb)

Abbreviations

CDP-Cho: Cytidyldiphosphatecholine; CDP-DAG: Cytidyldiphosphate diacylglycerol; CDP-Etn: Cytidyldiphosphate-ethanolamine; Cho: Choline; ChoP: Choline phosphate; Etn: Ethanolamine; EtnP: Ethanolamine phosphate; OMM: Outer mitochondrial membrane; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PS: Phosphatidylserine

Acknowledgements

We would like to acknowledge the anonymous reviewers for their work on this paper.

Funding

This research was supported by the International Science & Technology Cooperation Program of China (2016YFE0112900), the National Key R & D Plan (2017YFC1600904), the National Natural Science Fund for Distinguished Young Scholar (31525020) and the Young Elite Scientist Sponsorship Program (2017QNRC001).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and ZM conceived and designed the experiment. JW, HX, CZ and TW performed the experiment and analyzed the data. JW, CY and ZM checked all the data. JW, CY and ZM wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 2 January 2019 Accepted: 12 April 2019
Published online: 29 April 2019

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