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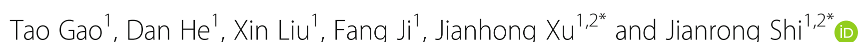
The pyruvate dehydrogenase kinase 2 (PDK2) is associated with conidiation, mycelial growth, and pathogenicity in *Fusarium graminearum*



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Abstract

Pyruvate dehydrogenase kinase (PDK) is a mitochondrial enzyme in a variety of eukaryotes, including the plant pathogen *Fusarium graminearum*. This enzyme can reduce the oxidation of glucose to acetyl-coA by phosphorylation and selectively inhibits the activity of pyruvate dehydrogenase (PDH), which is a kind of pyruvate dehydrogenase complex (PDC). In this study, we investigated the *F. graminearum* pyruvate dehydrogenase kinase encoded by *FgPDK2*, which is a homologue of *Neurospora crassa PDK2*. The disruption of the *FgPDK2* gene led to several phenotypic defects including effects on mycelial growth, conidiation, pigmentation, and pathogenicity. The mutants also showed decreased resistance to osmotic stress and cell membrane/wall-damaging agents. The *FgPDK2* deletion mutant exhibited reduced virulence. All of these defects were restored by genetic complementation of the mutant with the complete *FgPDK2* gene. Overall, the results demonstrated that *FgPDK2* is crucial for the growth of *F. graminearum* and can be exploited as a potential molecular target for novel fungicides to control Fusarium head blight caused by *F. graminearum*.

Keywords: *Fusarium graminearum*, Pyruvate dehydrogenase kinase, Vegetative differentiation, Reactive oxygen species (ROS)

Introduction

Fusarium head blight (FHB) caused by *Fusarium* species is an economically devastating disease of wheat and other small grain cereal crops around the world (Mcmullen et al. 1997). The toxin produced by *F. graminearum* also leads to a great risk to human and animal health and food safety (Kleter and Marvin 2009). Despite the high economic impact of FHB, there is currently no effective management strategy that does not rely on fungicides. Therefore, a better understanding of fungal development is helpful to develop new fungicides and new effective strategies to control FHB, which is important for wheat production and safety.

In general, biological cells continuously monitor their internal and external environments and sense signals for proliferation, differentiation, stagnation, or death, incorporating many exogenous foreign signals to control the possible progression or suspension of some or all cell activity. In eukaryotes, when the external environment changes or internal growth and development begin, the phosphorylation-dephosphorylation cycle is the main mechanism by which pathways are changed (Dickman and Yarden 1999). In most organisms, pyruvate is important as the end product of glycolysis (Papagianni

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2012) and can be metabolized by the pyruvate dehydrogenase complex (PDC). In mammals, the PDC can catalyze pyruvate metabolism to generate acetyl CoA through pyruvate decarboxylation; the main components of the PDC include three enzymes: pyruvate dehydrogenase (PDH, E1), dihydrothiocinamide acetyl transferase (E2) and dihydrothiocinamide dehydrogenase (E3) (Sugden and Holness 2011). The regulation of PDC activity is mainly achieved through the phosphorylation (inhibition) and dephosphorylation (activation) of serine residues at sites 293, 300 and 232 of the E1 alpha subfamily in PDH (Dickman and Yarden 1999). Pyruvate dehydrogenase kinase (PDK) is a kind of mitochondrial enzyme that can reduce the oxidation of glucose to acetyl-coA by phosphorylation and selectively inhibits the activity of PDH. At the same time, this step causes an increase in glycolysis and affects the oxidation of glucose (Roche and Hiromasa 2007). The research on PDK in mammals is extensive, and the activity of PDH complex enzymes can directly regulate the addition of pyruvate in the tricarboxylic acid cycle. In *Saccharomyces cerevisiae*, the conversion of pyruvate to acetyl-coA is accomplished by two PDKs. One regulates the phosphorylation of Pda1p ser133 residues, and the other regulates the phosphorylation of alpha subunits in PDCs (Krause-Buchholz et al. 2006). There are two PDHKs (Pkp1p and Pkp2p) in *S. cerevisiae* and deletion of them results in defects during growth in the presence of acetate and ethanol as carbon sources (Steensma et al. 2008). This phenomenon indicates that the PDHKs in *S. cerevisiae* may cause a predicted aborted carbon utilization cycle (Steensmays 1996). The PDHK in *A. nidulans*, PkpC (AN6207) was shown to be important for the secretion of cellulose and cellulose in the process of growth (Ries et al. 2018).

In *F. graminearum*, there are two PDKs (PDK1 and PDK2) with sequence similarity to the *S. cerevisiae* PDH kinases, sharing 26 and 29% amino acid identity with PKP1 and PKP2, respectively. The deletion of *FgPDK1* causes reduced growth on minimal medium supplemented with sucrose. The fungal morphology, conidiation and pathogenicity of *FgPDK1* deletion mutants also changed greatly compared with those of the wild-type strain (Gao et al. 2016). The objective of this study was therefore to explore the biological functions of another putative PDH kinase, FgPDK2. Genetic evidence indicated that FgPDK2 is essential for fungal growth and sexual reproduction, and fungal pathogenesis.

Materials and methods

Strains and culture conditions

The parental strain used for generating deletion mutants was PH-1, which is the standard strain of *F. graminearum* (Wang et al. 2019). Three different kinds of media (PDA, MM and CM) were used to analyze the colony morphology, and mycelial growth was measured with the cross-crossing method previously described (Gao et al. 2016). To evaluate the role of the *FgPDK2* gene in conidia production capacity, we inoculated ten 5-mm plugs of mycelia growth on PDA (3-day-old colony) into 100 ml MBB liquid medium (1.5% mung bean) with shaking at 180 rpm at 25 °C for 5 days (Chen and Zhou 2009). The capacity of conidia germination was analysed with the medium of YEPD (1% yeast extract, 2% peptone, 2% glucose). The medium used for fungal RNA mycelium sample collection was GYEP (5% glucose, 0.1% yeast extract, and 0.1% peptone) (Zheng et al. 2014).

Sequence analysis

The BLASTP algorithm was used for querying the sequence of the *PKP2* gene in *Saccharomyces cerevisiae* S288C (*NCBI* accession number NP_011456.5). The *PKP2* gene was used as the homologous sequence for searching the sequence of the putative mitochondrial protein kinase gene *FgPDK2* (FGSG_07381.3) in the database of *F. graminearum* (http://fungidb.org/fungidb/). Clustal W was used for the orthologues aligned of PDK2 amino acid sequences in different species. And MEGA v. 4.0.2 software was used for phylogenetic analysis by the method of neighbour-joining (Kumar et al. 2008).

DNA and RNA extraction

The CTAB (hexadecyl trimethyl ammonium bromide) method was used to extract mycelia genomic DNA (Zhang et al. 2009) and an RNeasy kit (Tian gen, China) was used to extract RNA from the samples that were cultured in YEPD and GYEP for 48 h. A PrimeScript® RT reagent kit (TaKaRa) was used to synthesize the first-strand cDNA, and the real-time detection system was a Roche Diagnostics system (Wang et al. 2010).

Deletion of the *FgPDK2* gene in *F. graminearum*

To analyze the function of *FgPDK2*, polyethylene glycol (PEG)-mediated protoplast transformation was used to construct the *FgPDK2* deletion mutant (ΔFgPDK2) with the genetic background of PH-1 (Gao et al. 2016). The strategy of double-joint PCR was used to construct the *FgPDK2* gene replacement vector (Yu et al. 2004). First, the 1.0 kb upstream (1.0-up) and 1.0 kb downstream (1.0-down) of *FgPDK2* gene were amplified from the parent strain PH-1 with the primers A1/A2 and A3/A4. Then, the hygromycin resistance gene (*HPH-tk* cassette) was initially amplified from the PtrpChptA-PItk plasmid with the primers of HTF/HTR. All of the primers are listed in supplementary data Table S1. To complement the full-length *FgPDK2* with the deletion mutants, the fragment of gene was inserted into the pYF11 plasmid

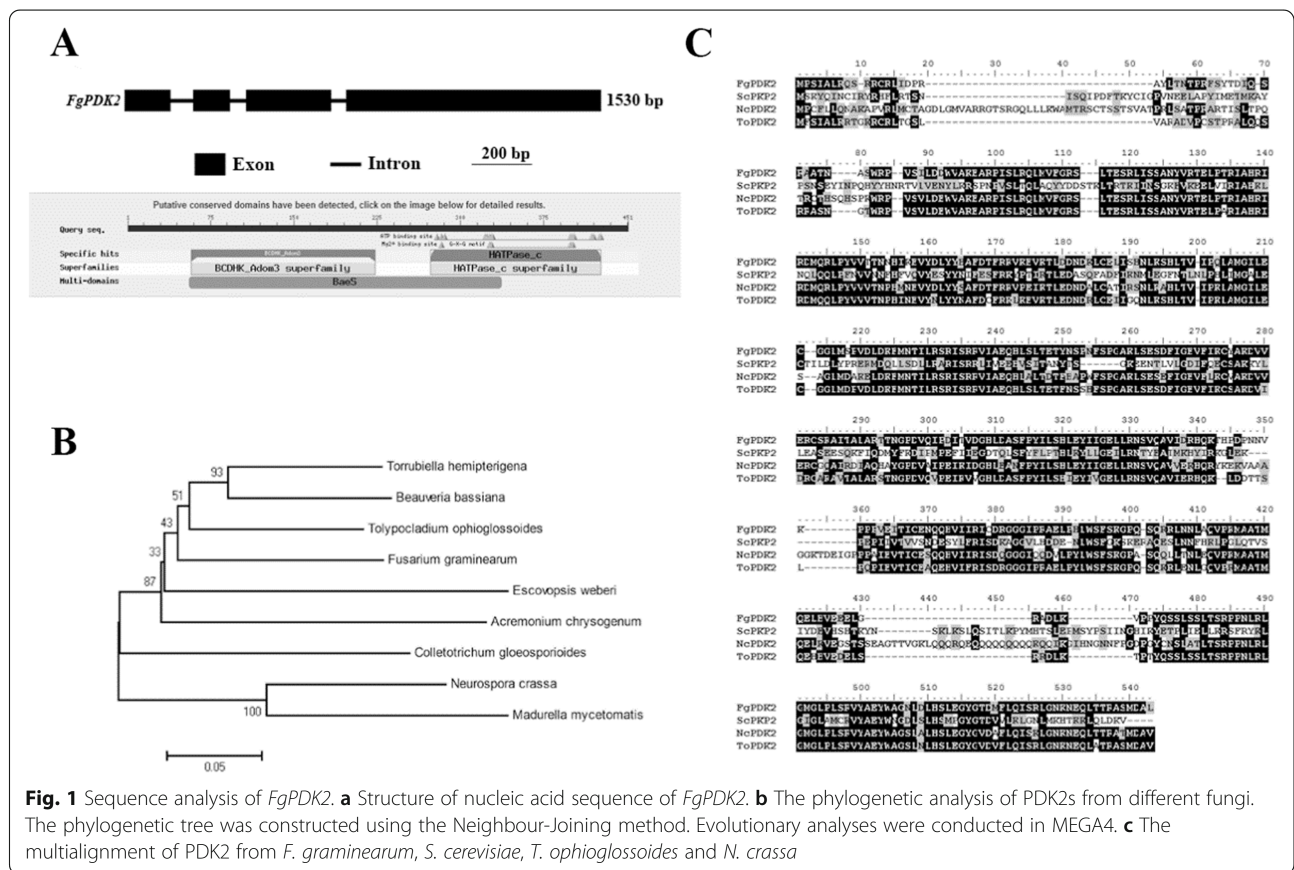


Fig. 1 Sequence analysis of *FgPDK2*. **a** Structure of nucleic acid sequence of *FgPDK2*. **b** The phylogenetic analysis of PDK2s from different fungi. The phylogenetic tree was constructed using the Neighbour-Joining method. Evolutionary analyses were conducted in MEGA4. **c** The multialignment of PDK2 from *F. graminearum*, *S. cerevisiae*, *T. ophioglossoides* and *N. crassa*

(Bruno et al. 2014), then the recombinant plasmid pYF11-*FgPDK2* was transformed into ΔFgPDK2. An AxyPreTP DNA Gel Extraction Kit (Axygen Biosciences, China) was used to purify the PCR products. Phenotypic characterization was firstly used to screen the resulting transformants, then the results of PCR amplification and Southern blotting confirmed the deletion mutants.

Cellular stress sensitivity evaluation

To determine the cell sensitivity of different strains, a 5-mm (diameter) mycelial plug was placed on PDA plates that were supplemented with the following solutes: osmotic stress inducers NaCl and KCl; the cell wall inhibitors caffeine and congo red; the cell membrane damager sodium dodecyl sulfate (SDS). The percentage of mycelial radial growth inhibition (RGI) was calculated using a previously described formula (Liu et al. 2019).

DON analysis

To determine the amount of DON produced by the mutants in vitro, five 5-mm (diameter) mycelial plugs were suspended in GYEP liquid medium and shaken at 28 °C in the dark for 7 days followed by filtration, drying and measurement of mycelium weight. DON was extracted and analysed using the protocol previously described by Ji et al. (2014). The HPLC-MS/MS system (Shimadzu 30A L C system coupled to a Triple Quad 6500 plus) was used to determine the quantity of DON with the methods which was previously described by Dong et al. (2016).

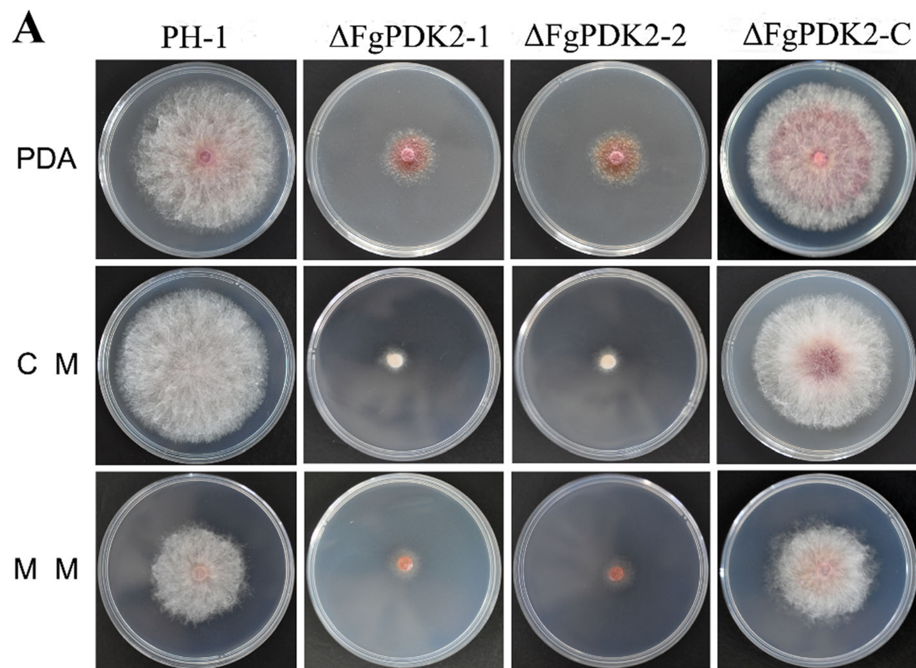
To analyze the expression levels of two trichothecene biosynthesis genes, *Tri5* and *Tri6*, ten mycelial plugs (5 mm diameter) from the edge of a 3-day-old colony of each strain were added into 100 ml GYEP and cultured for 48 h at 28 °C in the dark. Total RNA was extracted from the mycelia of each sample, and quantitative real-time PCR assays was used to determine the expression levels of *Tri5* and *Tri6*.

Pathogenicity in fresh tomatoes

To analyze the effect of *FgPDK2* on pathogenicity, mycelia of all strains were inoculated to fresh tomatoes (Di Pietro et al. 2001). After inoculation, the tomatoes were cultivated with 12 h of light per day for 5 days at 30 °C followed by measurement of lesions. . Each mutant or strain was represented by three replicate fruits, and the experiment was performed three times.

Statistical analysis

At least three replicated measurements were used to calculate the mean standard deviation (SD) and data are



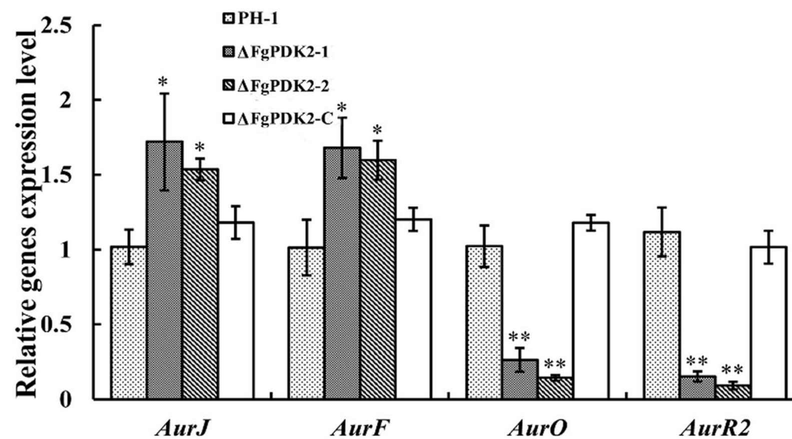


Fig. 2 Effect of *FgPDK2* deletion on *F. graminearum* colony morphology and pigment production. **a** The wild-type strain (PH-1), *FgPDK2* deletion mutant (ΔFgPDK2), and complemented strain (ΔFgPDK2-C) were grown on PDA, CM, and MM for 4 days at 25 °C. **b** Relative expression levels of four pigment-formation genes (*FgAurJ*, *FgAur*, *FgAurO*, and *FgAurR2*). The error bars in each column represent the standard deviation (SD) from three independent experiments; **P* < 0.05 or ***P* < 0.01 by Student's t-test

presented as the mean ± SD. The statistical comparison between wild-type parent PH-1 and deletion mutants were evaluated by SD of variance (ANOVA) with the software package SPSS 2.0.

Results

Identification of the *FgPDK2* gene in *F. graminearum*

The full sequence of *FgPDK2* gene (FGSG_07381.3) was obtained from the *F. graminearum* genome sequence which was deposited in the FungiDB Institute Database (https://fungidb.org/fungidb/). There are three introns in the *FgPDK2* gene. The first intron is located between the 150th and 222th nucleotides and the length is 73 bp; the second intron is located between the 343rd and 393rd nucleotides and the length is 51 bp; the third intron is located between the 677th and 726th nucleotides and the length is 50 bp (Fig. 1a). The deduced amino acid sequence of FgPDK2 from *F. graminearum*

Table 1 Biological properties of the *F. graminearum* deletion mutant ΔFgPDK2, the complemented strain ΔFgPDK2-C, and the wild-type strain PH-1*

Strain	Growth rate in vitro (mm/day)			Conidia produced in vitro (× 10^5/ml)
	PDA	CM	MM	
PH-1	20.78 ± 0.17a	21.22 ± 0.45a	13.77 ± 0.47a	1.77 ± 0.42a
ΔFgPDK2–1	5.36 ± 0.33b	4.28 ± 0.21b	3.11 ± 0.32b	0b
ΔFgPDK2–2	6.18 ± 0.14b	5.28 ± 0.19b	3.27 ± 0.23b	0b
ΔFgPDK2-C	20.44 ± 0.34a	21.11 ± 0.17a	13.67 ± 0.60a	1.71 ± 0.21a

* Values are the means (± standard error) of three replicates. Means in a column followed by the same letter are not significantly different according to the LSD test at $P < 0.05$

shares 79 and 70% identity with ToPDK2 of *Tolypocladium ophioglossoides* (KND93509.1) and NcPDK2 of *Neurospora crassa* (XP_961185.2), respectively (Fig. 1b), and FgPDK2 shares high similarity with other fungal PDK2 proteins (Fig. 1c).

Involvement of *FgPDK2* in mycelial morphogenesis and conidial formation of *F. graminearum*

To determine the cellular function of *FgPDK2*, we used the double-joint PCR approach to generate a gene replacement construct and transformed it into the PH-1 strain. The two deletion mutants ΔFgPDK2 and the complementary mutant ΔFgPDK2-C were confirmed by PCR and Southern blot analyses (Supplemental data Fig. S1).

The deletion mutants lacking *FgPDK2* (ΔFgPDK2–1 and ΔFgPDK2–2) showed different growth patterns on three different kinds of medium (PDA, CM and MM) compared with the wild-type strain PH-1 and complemented mutant strains (ΔFgPDK2-C) (Fig. 2a and Table 1). The deletion mutants ΔFgPDK2 grew significantly more slowly than the wild-type strain and did not produce any hyphae on these three kinds of media. The expression levels of the yellow pigment formation genes *AurO* and *AurR2* were significantly downregulated in the deletion mutants compared with those in the wild-type strain PH-1 and complemented mutant strains (ΔFgPDK2-C), while the expression levels of the red pigment-formation genes *AurJ* and *AurF* were slightly upregulated (Fig. 2b). The results of microscopy examination showed that the deletion of *FgPDK2* changed the morphology of aerial hyphae compared with that of the wild-type strain PH-1 (Fig. 3). In the process of asexual reproduction in *F. graminearum*, the ability to produce conidia is important. We found that the ΔFgPDK2 mutants failed to produce conidia compared to the wild-type and complemented strains (Table 1). These results showed that *FgPDK2* is required for conidial formation and mycelial growth.

Involvement of *FgPDK2* in the cell membrane permeability of *F. graminearum*

To ascertain the possible role of *FgPDK2* in the cell membrane and cell wall integrity of *F. graminearum*, the deletion mutants ΔFgPDK2 were exposed to four stress-inducing reagents: osmotic stressors (NaCl and KCl), cell membrane disrupter (SDS) and cell wall inhibitor (Congo red). Sensitivity to all those solutes was significantly greater in ΔFgPDK2 than in the parental strain PH-1 or in the complemented strain. There were no significant changes in these cellular stresses between the *FgPDK2*-deficient mutants and the PH-1 strain (Fig. 4). The results indicated that *FgPDK2* is involved in the regulation of cell membrane and cell wall integrity in *F. graminearum.*

Endogenous reactive oxygen species (ROS) is an important indicator of the extent of cell membrane damage and here we used specific probe DCFH-DA to detect it. We found that ROS in the ΔFgPDK2 mutants was increased compared to those in wild-type and complemented strains (Fig. 5). These results suggested that *FgPDK2* may be an important regulator of the integrity of cell membranes and walls in the stress responses of *F. graminearum.*

Involvement of *FgPDK2* in virulence and DON biosynthesis of *F. graminearum*

To explore the function of the *FgPDK2* gene in pathogenicity, we inoculated the wild-type and mutant strains to the tomatoes. The mutants lacking *FgPDK2* showed a

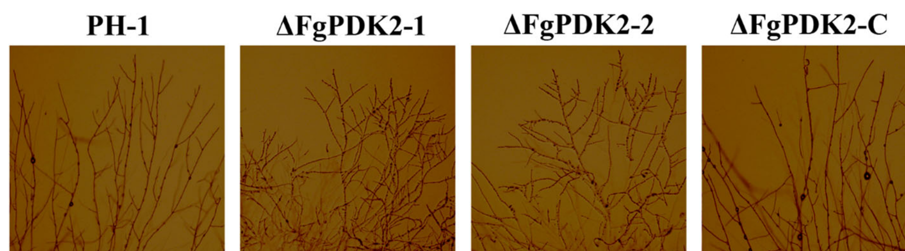


Fig. 3 Effect of *FgPDK2* deletion on *F. graminearum* hyphae morphology by light micrographs

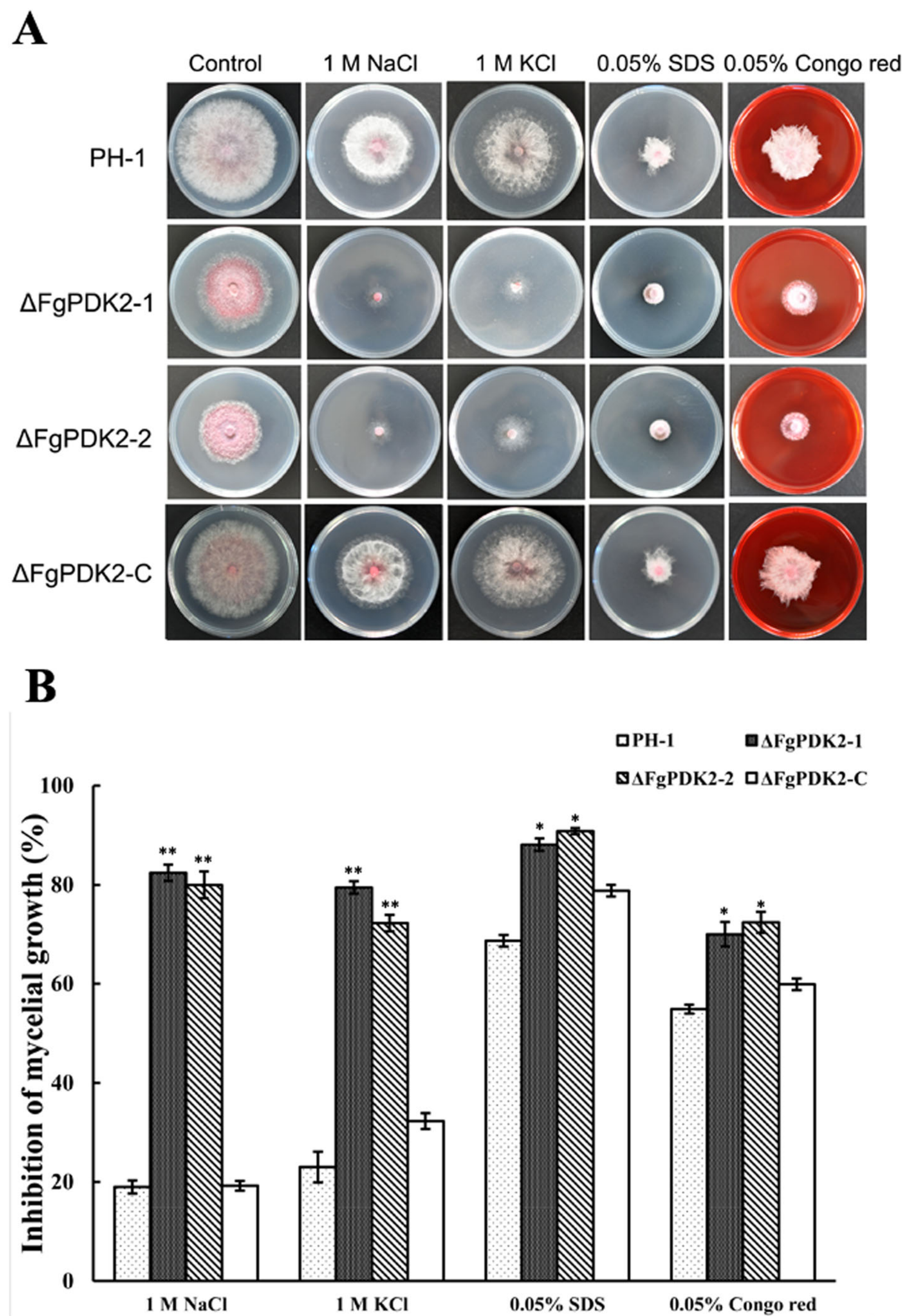


Fig. 4 Stress response of the wild-type strain PH-1, the *FgPDK2* deletion mutant (ΔFgPDK2), and the complemented strain (ΔFgPDK2-C). The strains were grown in medium containing different chemicals as indicated. Then the photographs were taken (**a**), and the inhibition of mycelial growth were measured (**b**). The error bars in each column represent the standard deviation (SD) from three independent experiments; *$P < 0.05$ or **$P < 0.01$ by Student's t-test

loss of pathogenicity, whereas the wild-type and complemented strains caused serious lesions (Fig. 6).

DON is a kind of mycotoxin that is produced by *F. graminearum*, and it is a virulence factor that helps the fungus establish and spread. Here we compared the ability of DON biosynthesis between the wild-type and mutant strains. In this study, the deletion mutants produced significantly ($P < 0.05$) less DON than the wild-type and complemented strains (Fig. 7a).

The expression levels of two related genes of DON biosynthesis (*Tri5* and *Tri6*) were significantly lower in the deletion mutants (ΔFgPDK2) than in the wild-type strain PH-1 and complemented strain (ΔFgPDK2-C) (Fig. 7b).

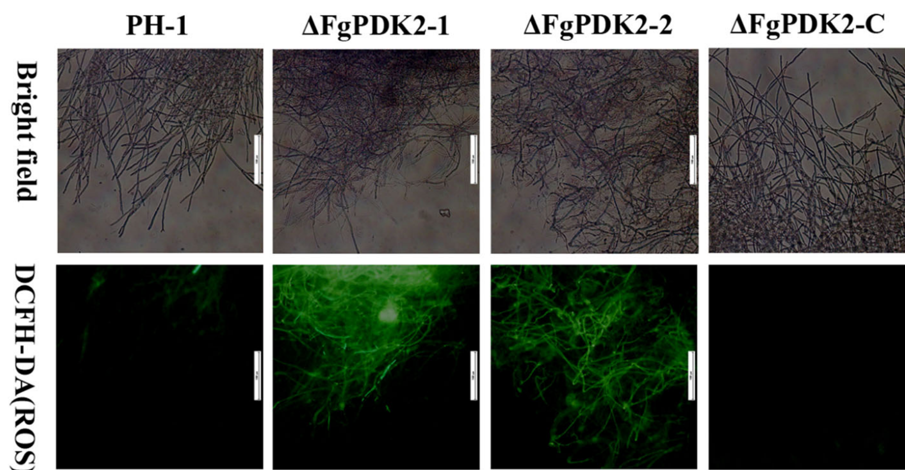


Fig. 5 The fluorescent staining of ROS in the mycelia of PH-1, the FgPDK2 deletion mutant (ΔFgPDK2), and the complemented strain (ΔFgPDK2-C). Mycelia were incubated with DCFH-DA followed by the fluorescent microscopic

Discussion

The mitochondrial pyruvate dehydrogenase complex (PDH) represents the core of carbon metabolism and is connected with two main energy-generating pathways: glycolysis and the TCA cycle, which ultimately lead to ATP production (Zhang et al. 2014). PDH activity is controlled by PDHKs and PDHPs, and these two enzymes play an important role in fungal development, pathogenicity and enzyme secretion (de Assis et al. 2015; Gao et al. 2016; Brown et al. 2013). Thus, the PDK enzymes involved in basic growth and essential pathways are potential desirable candidates to develop fungicides.

In this study, the hypothetical pyruvate dehydrogenase kinase PDK2 cloned from *F. graminearum* was identified on the basis of high sequence homology with other PDKs and the presence of BCDHK domains. The conservation of the PDK protein indicates that the kinase enzyme is an important regulator of metabolism and that its mechanism has been conserved throughout evolution. In mammalian cells, phosphorylation is performed by one or more tissue-specific kinases (PDHK1–4) (Sugden et al. 2000). Pyruvate dehydrogenase complex (PDC) activity is crucial for maintaining blood glucose and ATP levels, which largely depend on the phosphorylation status determined by pyruvate dehydrogenase kinase (PDK) isoenzymes (Jeong et al. 2012). Malfunctions of the pyruvate dehydrogenase complex have been linked to several diseases, the most prominent of which is lactic acidosis (Brown et al. 1994). The PDK complex has been studied thoroughly in several eukaryotes including a number of mammals, but the situation in fungi is less clear. Therefore, this study investigated that defects in pyruvate dehydrogenase kinase affect fungal development in *F. graminearum*. For functional analysis of this gene, disruptions were generated as described in materials and methods section. Basically, the PDK gene was replaced by an HPH cassette. The *FgPDK2* deletion mutants were viable but exhibited various defective developmental characteristics.

Studies have confirmed that the PDK complex of *S. cerevisiae* (both Pkp1 and the YGL059w product) is located in the mitochondria and that several amino acids (leucine, valine, isoleucine and arginine) occur (partly) in the mitochondria. In this study, the mutants exhibited substantial reductions in the formation of aerial mycelia and the production of conidia and perithecia. These changes evidently resulted in a reduced ability to infect hosts and cause disease. In the present study, the increased sensitivity to osmotic stresses mediated by the ΔFgPDK2 mutant suggested that *FgPDK2* is important for maintaining the integrity and stability of cell membranes. Under external stress conditions, cells produce a large amount of reactive oxygen species, which in turn cause lipid peroxidation damage in cell membrane. The PDK1 deletion of mutant of yeast showed high sensitivity to oxidative stress which was induced by hydrogen peroxide (Altintaş et al. 2015). This phenomenon was similar to that observed in the PDK2 deletion mutant in this study, which led to the accumulation of ROS.

Based on the foregoing, we may conclude that*FgPDK2* may protect the cell membranes from stress by reducing reactive oxygen species. PDK2's ability to scavenge reactive oxygen species may be attributed to lower mitochondrial respiration (Newington et al. 2012).

The regulatory proteins directly associated with DON synthesis in *F. graminearum* are controlled by *Tri* genes (Gardiner et al. 2009). Among these genes, Tri5 and Tri6 are the most important related to DON biosynthesis (Proctor et al. 1995). In this study, the reduced

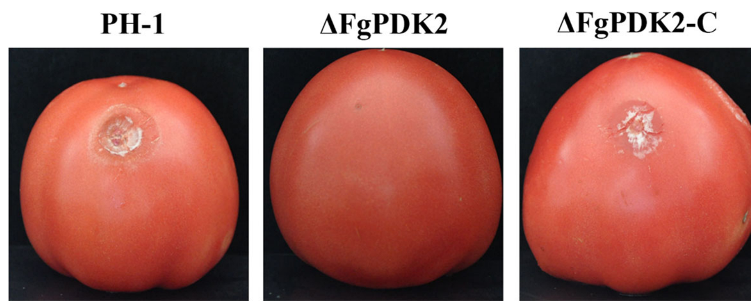


Fig. 6 Pathogenicity assays for *F. graminearum* strain PH-1, the *FgPDK2* deletion mutant (ΔFgPDK2) and the complemented strain (ΔFgPDK2-C). Lesions caused by strains on tomato fruits. Lesions were photographed after 5 days at 100% RH at 30 °C

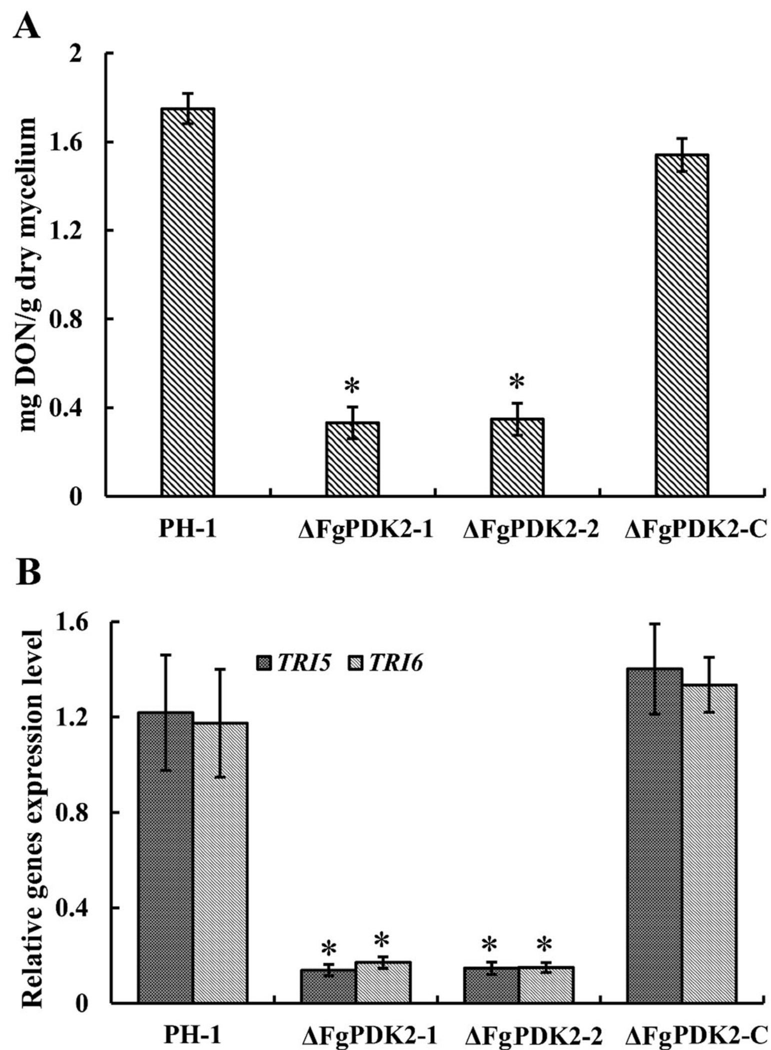


Fig. 7 Deoxynivalenol (DON) production (**a**) and relative expression of *Tri5* and *Tri6* (**b**) in PH-1, the *FgPDK2* deletion mutant (ΔFgPDK2), and the complemented strain (ΔFgPDK2-C). The error bars in each column represent the standard deviation (SD) from three independent experiments; *$P < 0.05$ by Student's t-test

pathogenicity of the *FgPDK2* deletion mutants may also have resulted from low trichothecene production together with reduced expression of the Tri genes. The reason for these findings may be that the infectious capacity and regulatory activity of the mutant hyphae were reduced.

Conclusions

In summary, the function of PDK2 in the plant pathogen *F. graminearum* was characterized. It was found that *FgPDK2* is important in the growth of *F. graminearum*. Deletion of *FgPDK2* can result in changes in mycelial morphogenesis, conidial formation and DON biosynthesis. Furthermore, *FgPDK2* is also crucial for the integrity of the cell membrane and cell walls. Therefore, current study demonstrated the vital role of PDK2 in this phytopathogen and all the results procured may provide a potential molecular target for novel fungicides design to control the FHB and reduce toxin pollution, which is very important for wheat production and safety.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s43014-020-00025-x.

Additional file 1: Table S1. Oligonucleotide primers used in this study and their relevant characteristics. **Fig. S1.** Schematic representation of the *FgPDK2*deletion strategy.

Abbreviations

FHB: Fusarium Head Blight; DON: Deoxynivalenol; PDA: Potato Dextrose Agar; MBB: Mung Bean Broth; YEPD: Yeast Extract Peptone Dextrose; GYEP: Glucose Yeast Extract Peptone; PDK: Pyruvate Dehydrogenase Kinase; HPLC-MS/MS: High-Performance Liquid Chromatography-Mass spectrometer/Mass spectrometer

Acknowledgements

Not applicable.

Authors' contributions

Tao Gao was involved in data acquisition, draft and critical revision of article, and final approval; Dan He was involved in data analysis; Xin Liu and Fang Ji were involved in data acquisition and data consulting; Jianrong Shi and Jianhong Xu conceived and designed the paper. The authors read and approved the manuscript.

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Availability of data and materials

Not applicable.

Competing interests

The authors declare no conflict of interest.

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