Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2018) 19(4), 909–921 DOI: 10.1111/mpp.12576

# The *PKR* regulatory subunit of protein kinase A (PKA) is involved in the regulation of growth, sexual and asexual development, and pathogenesis in *Fusarium graminearum*

CHAOQUN LI[1], YONGHUI ZHANG[2], HUAN WANG[1], LINGFENG CHEN[1], JU ZHANG[1], MANLI SUN[1], JIN-RONG XU[2] AND CHENFANG WANG[1,*]

[1]State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China
[2]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA

## SUMMARY

*Fusarium graminearum* is a causal agent of wheat scab disease and a producer of deoxynivalenol (DON) mycotoxins. Treatment with exogenous cyclic adenosine monophosphate (cAMP) increases its DON production. In this study, to better understand the role of the cAMP–protein kinase A (PKA) pathway in *F. graminearum*, we functionally characterized the *PKR* gene encoding the regulatory subunit of PKA. Mutants deleted of *PKR* were viable, but showed severe defects in growth, conidiation and plant infection. The *pkr* mutant produced compact colonies with shorter aerial hyphae with an increased number of nuclei in hyphal compartments. Mutant conidia were morphologically abnormal and appeared to undergo rapid autophagy-related cell death. The *pkr* mutant showed blocked perithecium development, but increased DON production. It had a disease index of less than unity and failed to spread to neighbouring spikelets. The mutant was unstable and spontaneous suppressors with a faster growth rate were often produced on older cultures. A total of 67 suppressor strains that grew faster than the original mutant were isolated. Three showed a similar growth rate and colony morphology to the wild-type, but were still defective in conidiation. Sequencing analysis with 18 candidate PKA-related genes in three representative suppressor strains identified mutations only in the *CPK1* catalytic subunit gene. Further characterization showed that 10 of the other 64 suppressor strains also had mutations in *CPK1*. Overall, these results showed that *PKR* is important for the regulation of hyphal growth, reproduction, pathogenesis and DON production, and mutations in *CPK1* are partially suppressive to the deletion of *PKR* in *F. graminearum*.

**Keywords:** autophagy, conidiation, DON, fungal pathogenicity, suppressor.

*Correspondence: Email: wangchenfang@nwafu.edu.cn

## INTRODUCTION

Fusarium head blight (FHB) or scab is one of the most important diseases of wheat and barley worldwide. One major causal agent of FHB is the homothallic ascomycete *Fusarium graminearum* (Bai and Shaner, 2004). The pathogen overwinters on plant debris and releases ascospores from perithecia in the spring to infect floral tissues of wheat, barley and other small grains (Goswami and Kistler, 2004). Under favourable environmental conditions, epidemiological outbreaks of FHB cause severe yield losses and reduce grain quality. *Fusarium graminearum* also produces asexual spores, known as conidia, on diseased plant tissues, but they are mainly for the infection and colonization of vegetative tissues later in the season. Because ascospores are the primary inoculum, sexual reproduction plays a critical role in the infection cycle of *F. graminearum* (Goswami and Kistler, 2004; Trail, 2009).

*Fusarium graminearum* is a producer of several harmful mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEA) (Audenaert *et al.*, 2014; Desjardins, 2003; Park *et al.*, 2016). As a trichothecene mycotoxin that is inhibitory to protein synthesis in eukaryotic organisms, DON is also toxic to plant cells. The *TRI5* trichodiene synthase gene was the first virulence factor characterized in *F. graminearum* (Alexander *et al.*, 2009; Bai *et al.*, 2002). Most of the trichothecene synthesis (*TRI*) genes responsible for DON production, including the two transcriptional regulators *TRI6* and *TRI10*, are in the same gene cluster with *TRI5* (Brown *et al.*, 2004). Several *TRI* genes, such as *TRI1*, *TRI4* and *TRI12*, encode proteins that localize to the toxisomes that are formed under DON-inducing conditions (Menke *et al.*, 2013). Various environmental or physiological conditions, such as pH, nitrogen sources and reactive oxygen species, are known to affect DON biosynthesis (Hou *et al.*, 2015; Jiang *et al.*, 2015; Merhej *et al.*, 2011), suggesting that *F. graminearum* can recognize and respond to these extracellular stimuli. Correspondingly, the cyclic adenosine monophosphate–protein kinase A (cAMP–PKA) pathway and all three mitogen-activated protein (MAP) kinase cascades have been shown to be required for DON production in *F. graminearum* (Hou *et al.*, 2002; Hu *et al.*, 2014; Zheng *et al.*, 2012), indicating that they must play critical roles in the regulation of DON biosynthesis in response to various environmental and host factors.

The cAMP–PKA pathway is well conserved in fungi and other eukaryotes (Lee *et al.*, 2003; Turra *et al.*, 2014). The inactive PKA complex consists of two regulatory and two catalytic subunits. The binding of the regulatory subunit with cAMP releases its inhibitory binding with the catalytic subunit. Free catalytic subunits of PKA activate various downstream targets that are important for growth, development, metabolism, stress responses and other cellular processes (Choi *et al.*, 2015; Li *et al.*, 2012; Turra *et al.*, 2014). In plant-pathogenic fungi, the cAMP–PKA pathway is important for the regulation of vegetative growth, sexual or asexual reproduction, and infection-related morphogenesis (Hu *et al.*, 2014; Voisey *et al.*, 2016; Yang *et al.*, 2016). In addition, cAMP signalling plays a critical role in the regulation of the production of mycotoxins, phytotoxins and other secondary metabolites (Hu *et al.*, 2014; Macheleidt *et al.*, 2016; Park *et al.*, 2016), such as ethylene biosynthesis in *Verticillium dahliae* and AT toxin production in *Alternaria alternata* (Tzima *et al.*, 2010; Xu *et al.*, 2011).

In *F. graminearum*, deletion of the *CPK1* gene encoding the major catalytic subunit of PKA significantly reduces the virulence and production of DON in cultures or infected plant tissues (Hu *et al.*, 2014). In contrast, deletion of the *PDE2* cAMP phosphodiesterase gene increases DON production (Jiang *et al.*, 2016). As in other eukaryotic organisms, intracellular cAMP is synthesized with adenylate cyclase, but degraded by cAMP phosphodiesterase in *F. graminearum*. Mutants deleted of the *FAC1* adenylate cyclase gene are almost blocked in DON production and plant infection in *F. graminearum* (Hu *et al.*, 2014). The intracellular cAMP level is elevated when cultured under DON-inducing conditions, and exogenous cAMP treatment stimulates DON biosynthesis in the wild-type (Jiang *et al.*, 2016). Treatments with exogenous cAMP also suppress the defects of the *Fgcap1* mutant in DON biosynthesis, suggesting a role of the adenylate cyclase-binding protein gene *FgCAP1* in the regulation of DON production via cAMP signalling (Yin *et al.*, 2017).

The only known intracellular target of cAMP in fungi is the regulatory subunit of PKA (PKR), which is also known to be important for the regulation of fungal growth, differentiation and pathogenesis (Fuller *et al.*, 2009; Staudohar *et al.*, 2002; Takano *et al.*, 2001). In this study, to further characterize the cAMP–PKA pathway in *F. graminearum*, we functionally characterized the *PKR* gene and identified spontaneous suppressor mutations in *CPK1*. Mutants deleted of *PKR* were viable, but showed severe defects in growth, conidiation and virulence. Conidia of the *pkr* mutant showed morphology and cell death defects. Deletion of *PKR* blocked sexual development, but increased DON production. Interestingly, the *pkr* mutant was genetically unstable and spontaneous suppressors with a faster growth rate were often produced on older cultures. Twelve of 67 *pkr* suppressor strains sequenced showed mutations in *CPK1*. Overall, these results show that *PKR* is important for hyphal growth, conidiation, sexual reproduction and plant infection, and mutations in *CPK1* are partially suppressive to the deletion of *PKR* in *F. graminearum*.

## RESULTS

### *PKR* is important for hyphal growth and nuclear distribution

The predicted *F. graminearum* gene FGRRES_09908 (named *PKR*) is orthologous to *BCY1* of *Saccharomyces cerevisiae*. It encodes a 399-amino-acid protein with typical structural components of regulatory subunits of PKA. To determine its function, we generated the *pkr* gene replacement mutants (Table 1) in the wild-type strain PH-1. Two *pkr* mutants (Table 1) were confirmed by Southern blot hybridization (Fig. S1, see Supporting Information).

The *pkr* mutants showed significantly reduced growth rates (Table 2) and formed compact colonies with limited, short aerial hyphae on potato dextrose agar (PDA) (Fig. 1A). In comparison with PH-1, the hyphal width of the *pkr* mutant was increased (Fig. 1B). The tip and intercalary hyphal compartments of *pkr* also contained more nuclei in each compartment than those of the wild-type (Fig. 1B), indicating that *PKR* plays a role in the regulation of cytokinesis and nuclear distribution during hyphal growth in *F. graminearum*.

**Table 1** The wild-type and mutant strains of *Fusarium graminearum* used in this study.

| Strains | Brief description | Reference |
|---|---|---|
| PH-1 | Wild-type | (Cuomo *et al.*, 2007) |
| P1 | *pkr* deletion mutant of PH-1 | This study |
| P3 | *pkr* deletion mutant of PH-1 | This study |
| C1 | *pkr/PKR* complemented transformant | This study |
| C2 | *pkr/PKR* complemented transformant | This study |
| C3 | *pkr/PKR*-GFP complemented transformant | This study |
| C4 | *pkr/PKR*-GFP complemented transformant | This study |
| G8-P2 | Transformant of P1 expressing GFP-*FgATG8* | This study |
| G8-P5 | Transformant of P1 expressing GFP-*FgATG8* | This study |
| G8-W3 | Transformant of PH-1 expressing GFP-*FgATG8* | This study |
| G8-W8 | Transformant of PH-1 expressing GFP-*FgATG8* | This study |
| H1 to H67 | Spontaneous suppressor strains of the *pkr* mutant P1 | This study |

**Table 2** Phenotypes of the *pkr* mutant in growth, conidiation and plant infection.

| Strain | Growth rate (mm/day)* | Conidiation ($\times 10^5$ conidia/mL) | Germination rate (%)† | Disease index‡ | DON (ppm) | |
|---|---|---|---|---|---|---|
| | | | | | Wheat§ | Rice¶ |
| PH-1 (WT) | $21.1 \pm 0.3^{A}$** | $11.9 \pm 1.4^{A}$ | $92.7 \pm 0.6^{A}$ | $13.0 \pm 1.1^{A}$ | $1896.8 \pm 84.3^{B}$ | $1465.3 \pm 427.0^{B}$ |
| P1 (*pkr*) | $5.9 \pm 0.3^{B}$ | $1.4 \pm 0.3^{B}$ | $14.1 \pm 3.8^{B}$ | $0.8 \pm 0.4^{B}$ | $6530.8 \pm 1034.2^{A}$ | $7369.4 \pm 655.9^{A}$ |
| C1 (*pkr/PKR*) | $19.2 \pm 0.2^{A}$ | $11.8 \pm 0.9^{A}$ | NA | $11.2 \pm 2.4^{A}$ | $2058.3 \pm 65.8^{B}$ | $1568.8 \pm 255.3^{B}$ |

*Average daily extension of colony radius/conidiation and standard deviation (mean ± standard deviation) were calculated from at least three independent measurements.

†A total of 300 conidia of each strain were counted for germination rate assays.

‡Diseased spikelets per wheat head were examined at 14 days post-inoculation (dpi). Mean and standard deviation were calculated from the results of three independent experiments.

§Flowering wheat heads were inoculated and infected wheat kernels were harvested for deoxynivalenol (DON) assays at 14 dpi.

¶Autoclaved rice grains were inoculated and cultured for 21 days, and then assayed for DON.

**The same letter indicates no significant difference. Different letters mark statistically significant differences ($P = 0.05$).

NA, not assayed.

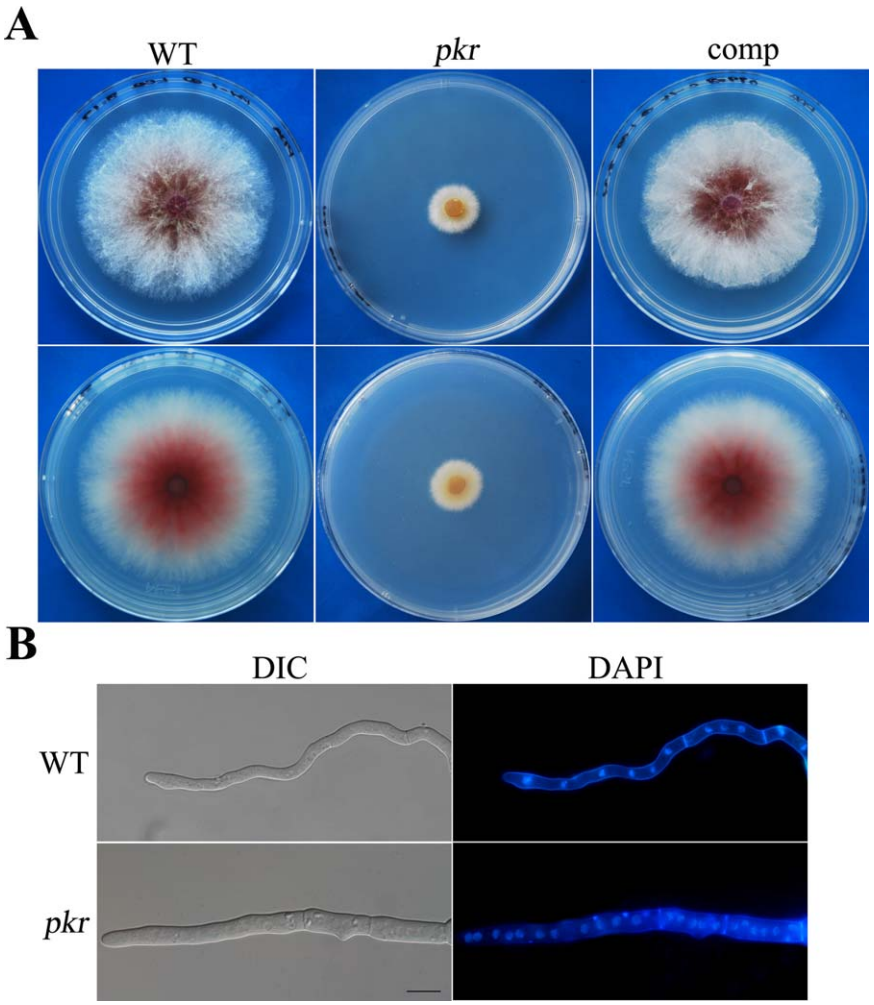


**Fig. 1** The *pkr* mutant was defective in growth and nuclear distribution. (A) Three day-old potato dextrose agar (PDA) cultures of the wild-type PH-1 (WT), *pkr* mutant P1 (*pkr*) and *pkr/PKR* complemented strain (comp). (B) 12-h germlings of PH-1 and *pkr* mutant were examined by epifluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI) and calcofluor white (CFW). DIC, differential interference contrast. Bar, 10 μm.

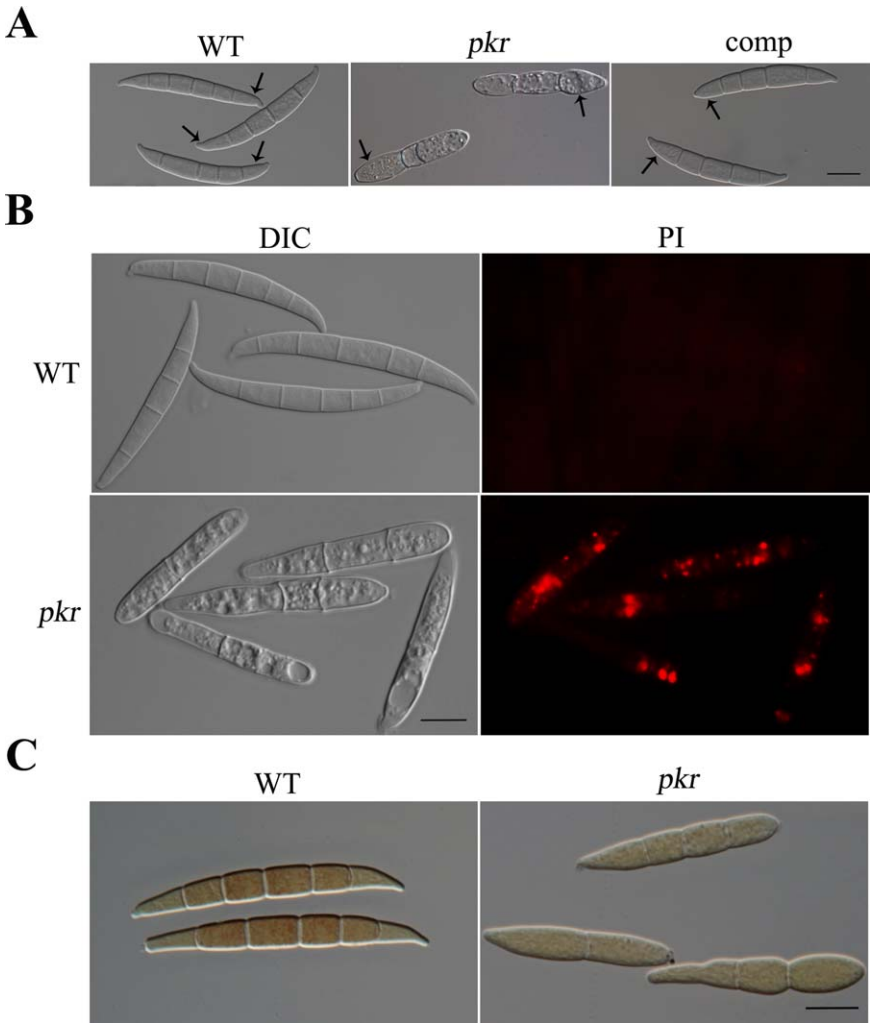


**Fig. 2** Defects of the *pkr* mutant in conidiation, cell viability and glycogen accumulation. (A) Conidia of wild-type PH-1 (WT), *pkr* mutant (*pkr*) and *pkr*/*PKR* complemented strain (comp) harvested from 5-day-old carboxymethyl cellulose (CMC) cultures. The *pkr* conidia appeared to be highly vacuolated or empty. Arrows indicate the foot cell. Bar, 10 μm.
(B) Five-day-old highly vacuolated *pkr* conidia were stained with 5 μg/mL propidium iodide (PI). Bar, 10 μm. DIC, differential interference contrast. (C) Conidia of PH-1 and *pkr* mutant harvested from 3-day-old CMC cultures were stained with KI/$I_2$ solution. Bar, 10 μm.

### The *pkr* mutant is defective in conidiogenesis and conidium viability

The *pkr* mutant also showed significantly reduced conidiation. In 5-day-old carboxymethyl cellulose (CMC) cultures, it produced less than 15% of conidia relative to the wild-type strain PH-1 (Table 2). In addition, the *pkr* mutant showed conidium morphology defects. Mutant conidia lacked typical foot cells and were shorter and less curved in comparison with those of the wild-type (Fig. 2A). Furthermore, many conidium compartments appeared to be empty in *pkr* conidia harvested from 5-day-old CMC cultures (Fig. 2A). When stained with propidium iodide (PI), most of these empty or highly vacuolated conidium compartments showed strong fluorescent signals, indicating that they were dead or dying (Fig. 2B). Under the same conditions, conidia of PH-1 were not stained with PI (Fig. 2B). Consistent with these observations, when incubated in YEPD (1% yeast extract, 2% peptone, 2% glucose), only 14.1% of *pkr* conidia germinated, but almost 100% of wild-type conidia produced germ tubes (Table 2), indicating that the majority of *pkr* conidia were no longer alive in 5-day-old cultures.

We then examined the conidia produced in younger cultures. Although they also showed morphological defects, *pkr* conidia harvested from 3-day-old CMC cultures showed normal viability and conidium compartments were not empty (Fig. 2C). However, microscopic observation showed that the *pkr* mutant was defective in glycogen accumulation in conidia. In 3-day-old CMC cultures, *pkr* conidia showed significantly reduced glycogen accumulation in comparison with those of the wild-type (Fig. 2C). These results indicate that *PKR* plays a role in the regulation of glycogen accumulation and viability of conidia in *F. graminearum*.

### Defects of the *pkr* mutant in autophagy

Because the *pkr* mutant was defective in conidium viability, we assayed its defects in autophagy. A green fluorescent protein (GFP)-*FgATG8* construct was generated and transformed into

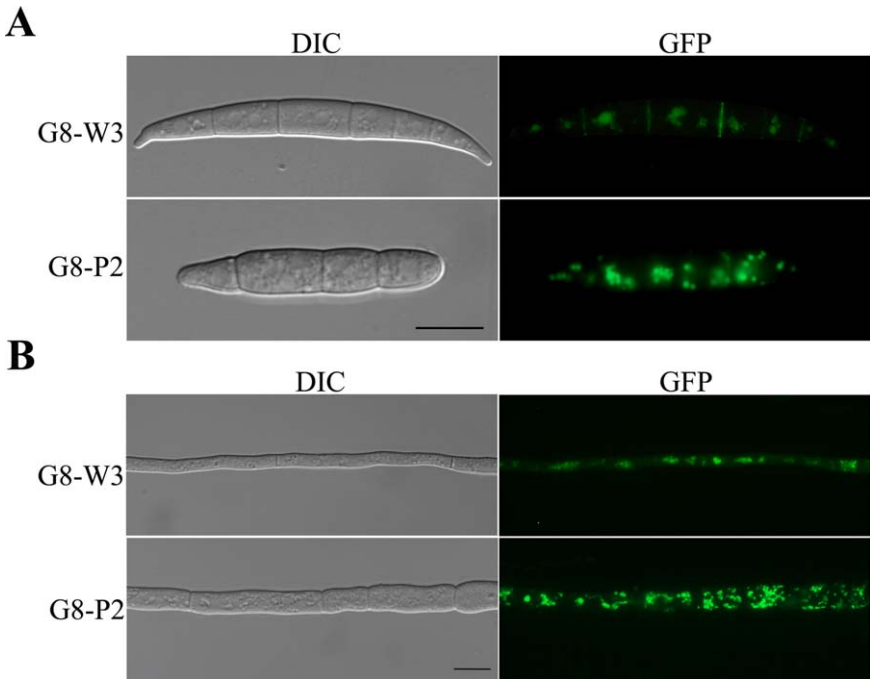


**Fig. 3** Assays for the localization of green fluorescent protein (GFP)-FgAtg8 fusion proteins. (A) Conidia harvested from 3-day-old carboxymethyl cellulose (CMC) cultures of transformants of the wild-type PH-1 and *pkr* mutant expressing the GFP-*FgATG8* construct were examined by differential interference contrast (DIC) and epifluorescence microscopy. (B) 24-h germlings of the same GFP-*FgATG8* transformants of the wild-type and *pkr* mutant were examined for the localization of GFP-FgAtg8 fusion proteins. Bar, 10 μm.

PH-1 and the *pkr* mutant. *FgATG8* encodes a ubiquitin-like protein required for the formation of autophagosomal membranes and autophagy (Josefsen *et al.*, 2012; Nakatogawa *et al.*, 2007). In the wild-type conidia harvested from 3-day-old CMC cultures, GFP-FgAtg8 fusion proteins were mainly localized to the autophagosomes and vacuoles (Fig. 3A). In comparison with the *pkr* mutant cultured under the same conditions, the wild-type contained far fewer autophagosomes in conidia (Fig. 3A). These results indicate that autophagic cell death may be responsible for conidium viability defects in the *pkr* mutant.

We also assayed the expression and localization of GFP-FgAtg8 fusion proteins in 24-h hyphae. In the wild-type background, weak GFP signals were observed in the cytoplasm and localization of GFP-FgAtg8 to the autophagosomes was rarely seen (Fig. 3B). Under the same conditions, the *pkr* mutant showed abundant punctate and globular autophagosomes with strong GFP-FgAtg8 signals (Fig. 3). These results indicate that deletion of *PKR* also results in defects in the regulation of autophagy during hyphal growth in *F. graminearum*.

## *PKR* is essential for sexual reproduction and plant infection

On self-mating carrot agar plates, the *pkr* mutant was sterile. No perithecia were observed in the mutant cultures at 14 days post-fertilization (dpf) or longer. Under the same conditions, the wild-type produced abundant perithecia and ascospore cirrhi (Fig. 4A). These data indicate that *PKR* is required for sexual development in *F. graminearum*.

In infection assays with flowering wheat heads, the wild-type caused typical head blight symptoms and showed a disease index of $13.0 \pm 1.1$ at 14 days post-inoculation (dpi). The *pkr* mutant caused scab symptoms only on the inoculated kernels. It failed to spread to neighbouring spikelets (Fig. 4B). The average disease index of *pkr* was $0.8 \pm 0.4$ (Table 2), which was significantly lower than that of the wild-type (Table 2). In infection assays with corn silks, unlike the wild-type, the *pkr* mutant failed to cause extensive discoloration beyond the inoculation site at 6 dpi (Fig. 4C). Therefore, *PKR* is important for plant infection in *F. graminearum*.

When the wild-type *PKR* allele was re-introduced into the *pkr* deletion mutant P1, the resulting *pkr*/*PKR* transformants were normal with regard to sexual reproduction and plant infection (Fig. 4). The *pkr*/*PKR* transformants were also complemented with regard to the defects in growth rate (Fig. 1) and conidiation (Fig. 2). These results indicate that the deletion of *PKR* is responsible for the defects observed in the *pkr* mutant.

## DON production is negatively regulated by *PKR*

Because DON is important for virulence, we assayed DON production in the *pkr* mutant. To our surprise, the *pkr* mutant showed increased DON production (approximately three-fold) in infested wheat kernels in comparison with the wild-type (Table 2). To confirm this observation, we assayed DON production in rice grain cultures as described by Bluhm *et al.* (2007) and Seo *et al.* (1996). Similar to the results with infested wheat grains, the *pkr* mutant showed increased DON production in rice grain cultures (Table 2),

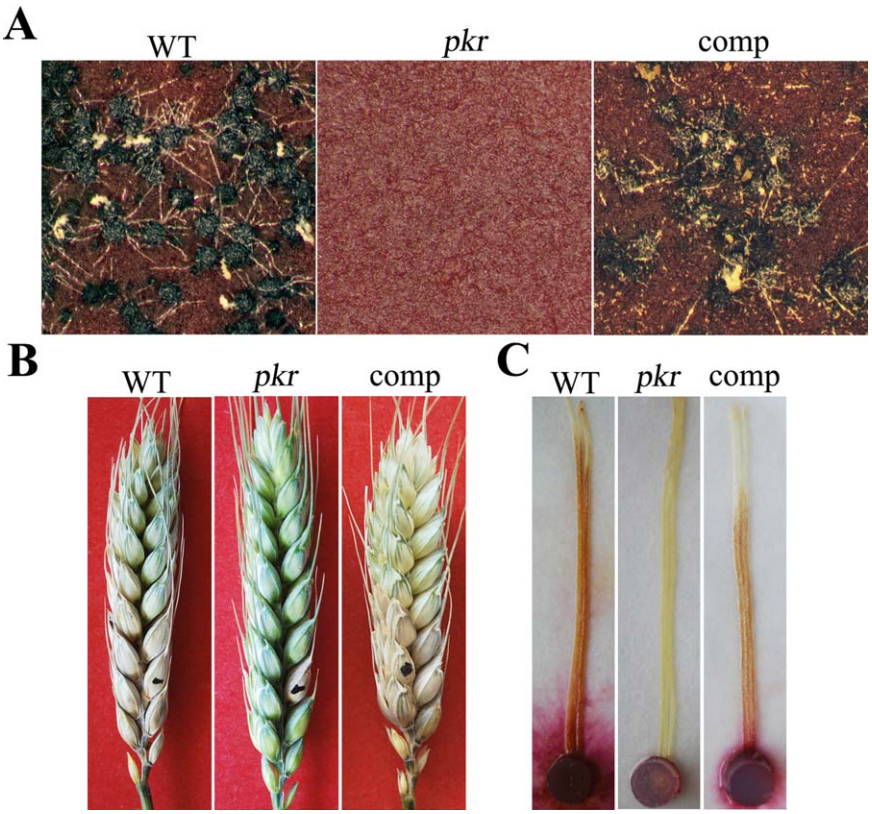


**Fig. 4** Defects of *pkr* in sexual reproduction and plant infection. (A) Carrot agar cultures of the wild-type PH-1 (WT), *pkr* mutant (*pkr*) and complemented *pkr/PPKR* transformant (comp) were examined at 14 days post-fertilization (dpf). Fertile perithecia with cirrhi were observed in PH-1 and the complemented transformant. (B) Flowering wheat heads were drop inoculated with conidia of the same set of strains and photographed at 14 days post-inoculation (dpi). Black dots mark the inoculated spikelets. (C) Corn silks inoculated with culture blocks were photographed at 6 dpi.

indicating that *PKR* plays a negative role in the regulation of DON biosynthesis.

We also assayed DON production in liquid trichothecene biosynthesis (LTB) medium after incubation for 5 days (Gardiner *et al.*, 2009b). The *pkr* mutant showed a seven-fold increase in DON production compared with the wild-type. To determine whether cAMP had any effect on the *pkr* mutant, we also assayed DON production in LTB cultures containing 4 mM cAMP. Exogenous cAMP increased DON production in PH-1 by approximately 37-fold, but had almost no effect on the *pkr* mutant (Fig. 5).

## Decreased expression levels of genes related to aurofusarin biosynthesis

In addition to DON, *F. graminearum* produces many other secondary metabolites, such as aurofusarins. The *pkr* mutant showed slightly reduced pigmentation in PDA and CMC cultures, suggesting a possible reduction in aurofusarin biosynthesis. To test this hypothesis, we assayed the expression levels of *GIP1*, *GIP2* and *PKS12* genes which are involved in aurofusarin biosynthesis (Kim *et al.*, 2005, 2006). In comparison with PH-1, the *pkr* mutant showed decreased expression of *GIP1*, *GIP2* and *PKS12* genes (60-, three- and five-fold, respectively) (Fig. S2, see Supporting Information). Therefore, it is likely that *PKR* plays various roles in the regulation of the biosynthesis of DON, aurofusarin and possibly other secondary metabolites in *F. graminearum*.

## Subcellular localization of PKR-GFP fusion proteins

To determine its subcellular localization, the *PKR*-GFP fusion construct was generated and introduced into the *pkr* mutant. The

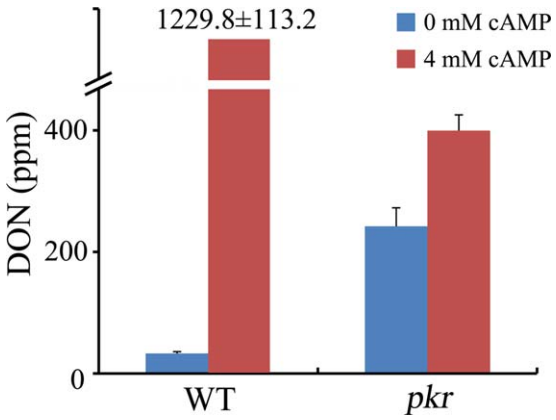


**Fig. 5** Defects of the *pkr* mutant in response to exogenous cyclic adenosine monophosphate (cAMP). Deoxynivalenol (DON) production in 5-day-old liquid trichothecene biosynthesis (LTB) cultures of wild-type PH-1 (WT) and the *pkr* mutant treated with or without 4 mM cAMP.

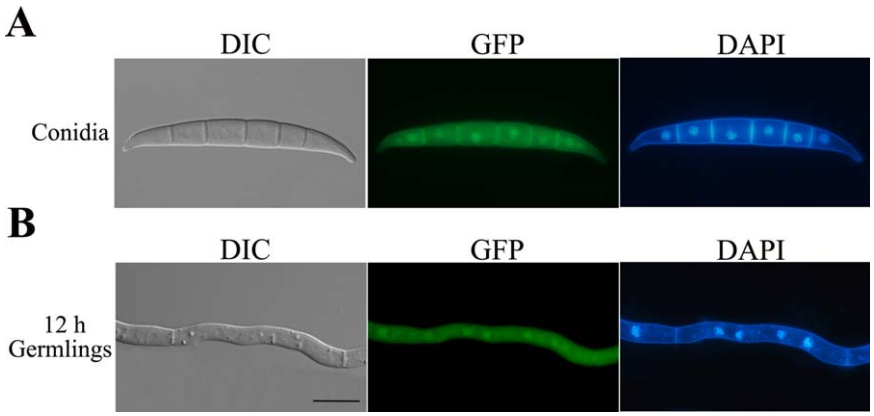


**Fig. 6** Subcellular localization of PKR-GFP fusion proteins. Conidia (A) and 12-h germlings (B) of the *pkr/PKR-GFP* transformant were examined by differential interference contrast (DIC) and epifluorescence microscopy. Green fluorescent protein (GFP) signals were observed in the nucleus and cytoplasm in conidia and 12-h germlings. Bar, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole.

resulting *pkr/PKR*-GFP transformant was normal with regard to hyphal growth and conidial size, indicating that fusion with GFP has no obvious effects on the function of *PKR*. When examined by epifluorescence microscopy, localization of PKR-GFP to the nucleus and cytoplasm was observed in conidia (Fig. 6A) and 12-h germlings (Fig. 6B). However, GFP signals were stronger in the nucleus, particularly in the conidia (Fig. 6).

## The *pkr* mutant is unstable and produces spontaneous suppressors with a faster growth rate

Interestingly, the *pkr* mutant was unstable. After incubation on PDA at 25 °C for 5 days or longer, fast-growing sectors derived from spontaneous mutations were often observed (Fig. 7A). In total, 67 suppressor strains with different growth rates and conidiation levels were isolated (Table S1, see Supporting Information). Only three, H1, H2 and H3, grew almost as fast as the wild-type strain (Table S1). Interestingly, these three suppressor strains also showed similar colony morphology to PH-1 (Fig. 7B). Nevertheless, conidiation was not fully recovered in any of these three strains. In suppressor H2, conidiation was increased to approximately 30% of the wild-type level, but suppressors H1 and H3 produced fewer conidia than the original *pkr* mutant (Table S1).

The remaining 64 suppressor strains still grew much more slowly than the wild-type strain. Their growth rates varied from 30% to 75% of that of PH-1 (Table S1). These suppressor strains also varied in colony morphology (Fig. 7B). Interestingly, a number of them, such as H12, produced as many or more conidia than the wild-type, although their growth rate was slower than that of PH-1 (Table S1). Differences among suppressor strains in growth rate and conidiation indicate that spontaneous mutations may have occurred in different genes functionally related to PKA.

## Mutations in *CPK1* are identified in 12 suppressor strains

To identify the genetic mutations responsible for the suppression of the *pkr* mutant, we selected three suppressor strains [H3, H7 and H14 with growth rates similar to, or 62.6% or 59.0% of PH-1 (Table S1)] to sequence 18 candidate genes (Table S2, see Supporting Information) orthologous to the downstream targets of PKA or genes functionally related to PKA in yeast. No mutation sites were detected in any of these candidate genes sequenced in suppressor H14. In suppressor strain H3, the non-synonymous mutation resulting in the E406A change in amino acid sequences was identified in the *CPK1* gene. Suppressor strain H7 also showed a mutation in *CPK1* that resulted in the H531R amino acid change. For both H3 and H7, no mutation was identified in the 17 other candidate genes sequenced (Table S2).

We then amplified and sequenced the *CPK1* gene from the remaining 64 suppressor strains. Ten showed mutations in *CPK1* (Table S1) at nine different sites (Fig. 8). One suppressor mutant showed the deletion of residues 310–315 beside the ATP-binding site, and two others showed frameshift mutations at P19 and L352, respectively (Fig. 8). Another two suppressor strains, H1 and H62, showed the same frameshift mutation at Y575 in the C′-terminal region downstream from the kinase domain (Fig. 8). All other suppressor strains showed non-synonymous mutations resulting in changes at amino acid residues that are well conserved in Cpk1 and its orthologues from filamentous fungi (Fig. 8). Eight suppressor strains revealed mutations in six different protein kinase subdomains, including two each in subdomains V and XI and one each in subdomains II, VI, VII and IX. One suppressor strain, H2, showed the D561N mutation at a well-conserved residue in the C-terminal region (Fig. 8). In summary, all the mutations identified in *CPK1* resulted in either frameshift mutations or non-synonymous mutations at well-conserved residues that probably affected PKA activities to various degrees.

## Increased expression of *CPK1* and *CPK2* in the *pkr* mutant is suppressed by suppressor mutations

To determine whether the deletion of *PKR* and mutation in *CPK1* affect the expression of the catalytic subunits of PKA, RNA samples were isolated from 12-h YEPD cultures of PH-1, the *pkr*

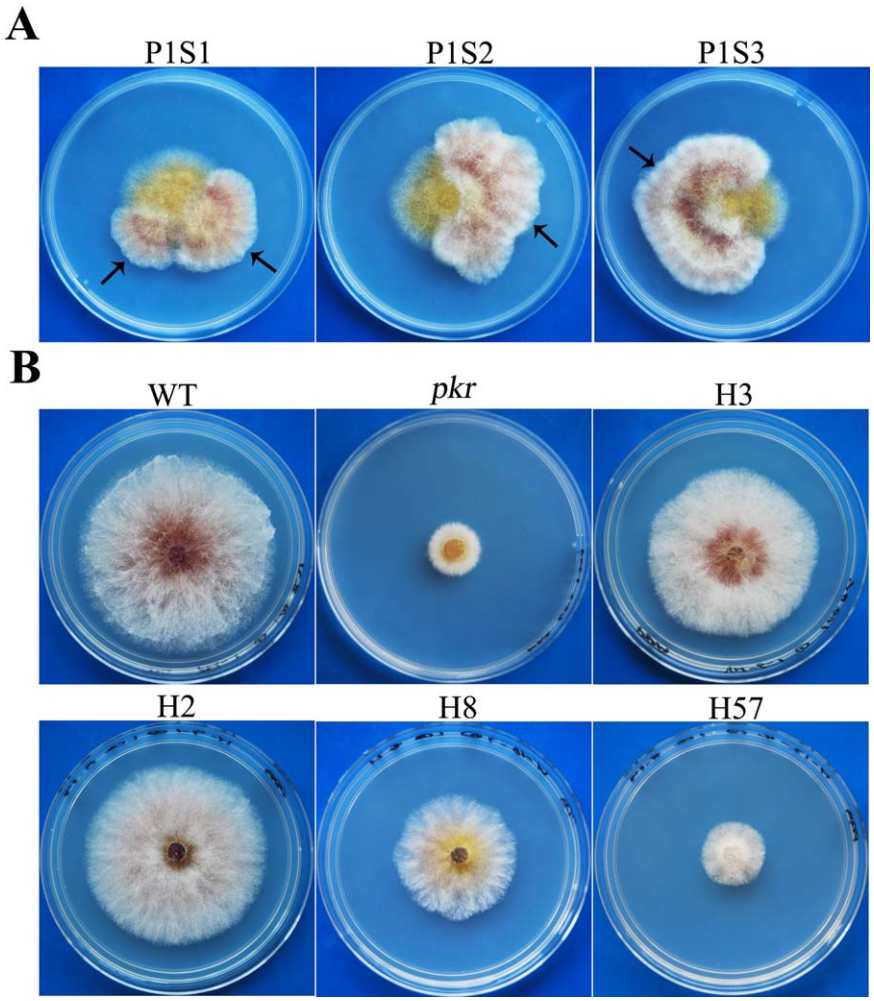


**Fig. 7** Spontaneous suppressors of the *pkr* mutant. (A) Six-day-old potato dextrose agar (PDA) cultures of the *pkr* mutant with fast-growing sectors (marked with arrows). (B) Three-day-old PDA cultures of four representative spontaneous suppressor strains with different growth rates. WT, wild-type.

mutant and 12 suppressors with mutations in *CPK1*. The expression levels of *CPK1* and *CPK2* were assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In comparison with the wild-type, *CPK1* expression was increased over 2.9-fold in the *pkr* mutant (Fig. S3, see Supporting Information). Although the expression level of *CPK2* was not as high as that of *CPK1*, it was also increased approximately two-fold. However, the expression levels of both *CPK1* and *CPK2* showed no significant difference between PH-1 and 12 suppressor strains (Fig. S3). Therefore, genetic mutations in these suppressor strains must be able to suppress the effect of *PKR* deletion on the expression of the PKA catalytic subunit genes in *F. graminearum*.

## DISCUSSION

Like its orthologues, Pkr has typical structural components of the regulatory subunit of PKA. In *F. graminearum*, the deletion of *PKR* was not lethal, but the *pkr* mutant showed severe defects in growth and conidiation. It formed compact colonies, which were similar to those of the *cpk1 cpk2* double mutants (Hu *et al.*, 2014). In *Aspergillus fumigatus* and *Colletotrichum lagenarium*, deletion of the *PKR* orthologue also results in a significant reduction in vegetative growth and conidiation (Takano *et al.*, 2001; Zhao *et al.*, 2006). In *Neurospora crassa*, the deletion mutant is not available, but a temperature-sensitive mutation in *MCB1* affects growth polarity at the restrictive temperature (Bruno *et al.*, 1996). In *Aspergillus niger*, PKR also regulates the growth polarity during submerged growth and is essential for conidiation (Staudohar *et al.*, 2002). Therefore, the regulatory subunit of PKA may play a conserved role in growth and asexual reproduction in filamentous ascomycetes.

The *pkr* mutant was significantly reduced in virulence. It failed to spread from the inoculated wheat kernels to neighbouring spikelets and caused only limited necrosis on corn silks. In *C. lagenarium*, although the *rpk1* mutants formed lesions on cucumber plants when inoculated through wounds, they were non-pathogenic on intact leaves (Takano *et al.*, 2001). The Δ*pkaR* mutant of *A. fumigatus* was also defective in virulence (Zhao *et al.*, 2006). In *Ustilago maydis*, the *ubc1* mutant colonized maize plants,

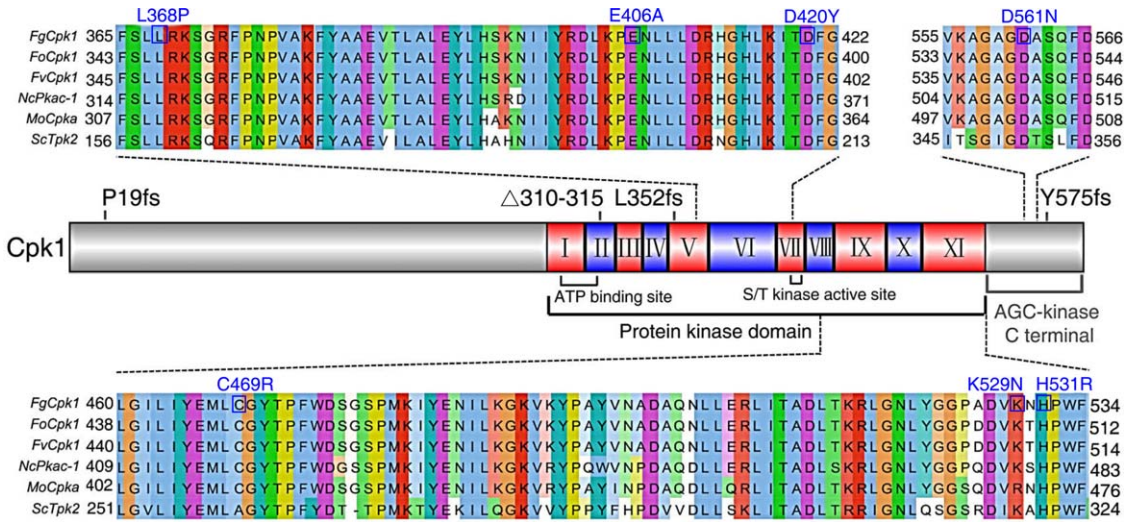


**Fig. 8** Schematic drawing of mutation sites identified in *CPK1*. Amino acid changes in *CPK1* identified in 12 suppressor strains are labelled above the schematic drawing of Cpk1 or sequence alignments of Cpk1 and its orthologues from *Fusarium oxysporum* (Fo), *Fusarium verticillioides* (Fv), *Magnaporthe oryzae* (Mo), *Neurospora crassa* (Nc) and *Saccharomyces cerevisiae* (Sc). The conserved amino acid residues with suppressor mutation in Cpk1 are labelled with blue boxes. The predicted ATP-binding site and the serine/threonine (S/T) kinase active site are labelled underneath the schematic drawing of Cpk1. I–XI, 11 protein kinase subdomains.

but failed to induce gall formation (Gold *et al.*, 1994, 1997). In *F. graminearum*, the reduced growth rate of the *pkr* mutant may contribute to its defects in pathogenesis. However, in *Cryptococcus neoformans*, an opportunistic human pathogenic fungus, the PKA regulatory subunit mutant *pka1* showed increased virulence and capsule production (D'Souza *et al.*, 2001). Therefore, the PKA regulatory subunit probably varies in its roles in regulating infection processes in different plant and animal fungal pathogens.

Conidia of the *pkr* mutant lacked a distinct foot cell and appeared to age rapidly. In 5-day-old CMC cultures, most of the *pkr* conidia were highly vacuolated and often had empty conidium compartments. Only 14.1% of these *pkr* conidia were alive, which is a significant reduction in comparison with the almost 100% germination rate in the wild-type. Assays for the expression and localization of GFP-FgAtg8 indicated that the *pkr* mutant showed increased autophagosome formation and autophagy in conidia harvested from 3-day-old conidia. In mammalian cells, the regulatory subunits of PKA (PKAR) and mTOR are involved in a common pathway regulating autophagy, and deletion of PKAR leads to autophagic deficiency by the activation of mTOR (Mavrakis *et al.*, 2006). In *S. cerevisiae*, elevated PKA activity also negatively regulates autophagy, and inactivation of the PKA pathway is sufficient to induce a robust autophagic response (Stephan *et al.*, 2009). However, in *F. graminearum*, deletion of *PKR* may activate or over-activate autophagy and result in cell death and empty compartments in conidia. It is likely that the PKA pathway is well conserved for the regulation of autophagy in humans, yeasts and filamentous fungi, although different organisms may differ in their regulatory mechanisms.

Interestingly, in comparison with the wild-type, the *pkr* mutant showed increased DON production in infected wheat kernels, rice grain cultures and LTB cultures. In *F. graminearum*, treatment with exogenous cAMP is known to stimulate DON biosynthesis (Jiang *et al.*, 2016). In comparison with the wild-type, the *pkr* mutant showed a defective response to cAMP treatment for increased DON production. One likely explanation is that the deletion of *PKR* may increase PKA activity, which, in turn, up-regulates the expression of *TRI* genes. Nevertheless, it remains possible that increased DON production in the *pkr* mutant may be related to its defects in the proper regulation of autophagy and secondary metabolism. The *pkr* mutant may be constitutively or over-activated in autophagy and the biosynthesis of DON and other secondary metabolites. The *pkr* mutant displayed slightly reduced pigmentation in PDA and CMC cultures (forming yellowish rather than reddish colonies) and showed reduced expression levels of *GIP1*, *GIP2* and *PKS12*, suggesting a possible reduction in aurofusarin biosynthesis. An earlier study has shown that these genes are negatively regulated by *CPK1* (Hu *et al.*, 2014). The biosynthesis of ZEA, an oestrogenic mycotoxin, is also positively regulated by *PKR* and negatively regulated by *CPK1* (Park *et al.*, 2016). These results indicate that catalytic subunits and regulator subunits of PKA play different roles in the regulation of various secondary metabolites. In *F. graminearum*, *CPK1* is known to regulate secondary metabolism in general (Hu *et al.*, 2014). It is not surprising that *PKR* also plays a broad role in the regulation of the biosynthesis of secondary metabolites.

The *pkr* mutant of *F. graminearum* was unstable and produced faster growing spontaneous suppressors that differed in growth

rate and conidiation. Spontaneous suppression mutations of PKA regulatory subunit mutants have been observed in other fungi, such as *A. niger* and *C. lagenarium* (Staudohar *et al.*, 2002; Takano *et al.*, 2001). Among the 67 suppressor strains isolated, 12 showed mutation in the *CPK1* catalytic subunit gene (Hu *et al.*, 2014). Four showed frameshift mutations and one exhibited a deletion of six conserved amino acid residues in the predicted ATP-binding sites. These mutations, in particular the deletion of residues 310–315 and frameshift mutations at P19 or L352, probably resulted in a null allele of *CPK1*. The detrimental effect of *PKR* deletion may be partially suppressed by mutations in *CPK1*, contributing to the majority of PKA activities in *F. graminearum* (Hu *et al.*, 2014).

All the other suppressor strains with mutations identified in *CPK1* exhibited non-synonymous mutations at well-conserved amino acid residues. Indeed, sequence alignment showed that all the non-synonymous mutation sites are conserved in yeast Tpk2 and its orthologues from filamentous fungi (Fig. 8). These mutations in *CPK1*, including L368P, E406A, D420Y, C469R, K529N, H531R and D561N (Fig. 8), also probably affect PKA activities. In qRT-PCR assays, these suppressor mutations suppressed the stimulatory effect of *PKR* deletion on the expression of *CPK1* and *CPK2*. E406 is in the serine/threonine (S/T) kinase active site which is important for the structure and function of PKA. L368, E406, D420, C469, K529 and H531 are also in the predicted kinase subdomains. In yeast, mutants carrying the T210W mutation in *TPK2* were non-viable in the presence of 5-fluoroorotic acid (5-FOA) (Kennedy *et al.*, 2008). Residue T210 interacts with adenosine within the nucleotide-binding pocket and adenosine enhances the overall stability of PKA (Herberg *et al.*, 1999). In *F. graminearum*, suppressor H66 showed the D420Y mutation in *CPK1*. Based on sequence alignment, D420 of Cpk1 is equivalent to D211 of *Tpk2*. Therefore, it is possible that the D420Y mutation in *CPK1* also affects its interaction with adenosine and PKA activity. Moreover, the lower expression level of *CPK1* in suppressor H66 also suggested that the D420Y mutation in *CPK1* was important for *CPK1* transcription. In addition, L368 and residues 310–315 are also located in the nucleotide-binding pocket based on the structural features of PKA in *S. cerevisiae* (Kennedy *et al.*, 2008). The L368P mutation and deletion of residues 310–315 may also affect ATP binding and PKA activities in *F. graminearum*.

In summary, our study showed that the *pkr* mutant exhibited pleiotropic defects in hyphal growth, conidiation, sexual development and virulence. Deletion of *PKR* also affected autophagy and cell death in conidia. Interestingly, *PKR* negatively regulates DON production, but positively regulates aurofusarin biosynthesis. Overall, our results confirmed the importance of the cAMP–PKA pathway in growth, development and pathogenesis, and showed the complexity of its regulatory mechanisms in secondary metabolism. Further characterization of the spontaneous suppressors of the *pkr* mutant will be helpful to define and clarify the regulatory networks related to cAMP signalling in *F. graminearum*.

## EXPERIMENTAL PROCEDURES

### Strains and culture conditions

The wild-type strain PH-1 (Cuomo *et al.*, 2007) and mutants of *F. graminearum* generated in this study are listed in Table 1. The growth rates on potato dextrose agar (PDA) plates, conidiation in liquid carboxymethyl cellulose (CMC) medium and sexual reproduction on carrot agar plates were assayed as described previously (Ding *et al.*, 2009; Zhou *et al.*, 2010). Protoplast preparation and transformation of *F. graminearum* were performed as described previously (Hou *et al.*, 2002). For transformant selection, hygromycin B (CalBiochem, La Jolla, CA, USA) and geneticin (Sigma, St. Louis, MO, USA) were added to final concentrations of 300 and 350 μg/mL, respectively, to the top agar. For DNA isolation, vegetative hyphae were harvested by filtration from liquid YEPD after incubation at 25 °C for 12 h.

### Generation of the *pkr* mutant

For the generation of the gene replacement construct by the split-marker approach, the 620-bp upstream and 437-bp downstream fragments of *PKR* were amplified with the primer pairs PKR/1F–PKR/2R and PKR/3F–PKR/4R (Table S3, see Supporting Information), respectively. The resulting PCR products were connected to the *hph* hygromycin phosphotransferase cassette by overlapping PCR and transformed into protoplasts of PH-1, as described previously (Zhou *et al.*, 2010). Hygromycin-resistant transformants were screened by PCR with the primers PKR/5F and PKR/6R (Table S3). Putative *pkr* mutants identified by PCR were confirmed by Southern blot analysis.

For complementation assays, the entire *PKR* gene and its promoter and terminator sequences were amplified with primers CM-*PKR*/F and CM-*PKR*/R (Table S3), digested with *Sma*I and *Sac*II, and cloned into the $NEO^R$ vector pHZ100 (Bluhm *et al.*, 2007). The resulting construct pPKR was transformed into protoplasts of the *pkr* mutant P1. The *pkr/PKR* transformants were verified by PCR and Southern blot analyses.

### Generation of the *PKR*-GFP transformant

To generate the *PKR*-GFP construct, the entire *PKR* gene was amplified with primers *PKR*-eGFP-F and *PKR*-eGFP-R, and cloned into *Xho*I-digested pFL2 by the yeast gap repair approach (Bruno *et al.*, 2004; Zhou *et al.*, 2011). The resulting *PKR*-GFP construct carrying the geneticin-resistant marker rescued from $Trp^+$ yeast transforms was verified by sequencing analysis and transformed into the *pkr* mutant P1. Transformants resistant to G418 were verified by PCR to carry the *PKR*-GFP construct and examined for GFP signals by epifluorescence microscopy.

### Plant infection and DON production assays

For infection with wheat cultivar Xiaoyang 22, conidia of PH-1 and mutant strains were harvested from CMC cultures by filtration and re-suspended to $10^5$ spores/mL, as described previously (Jiang *et al.*, 2016). For each flowering wheat head, the fifth spikelet from the base was inoculated with 10 μL of conidial suspension, as described previously (Ding *et al.*,

2009; Gale *et al.*, 2007). Spikelets with typical wheat scab symptoms were examined at 14 days post-inoculation (dpi) to estimate the disease index (Wang *et al.*, 2011), and diseased wheat kernels were assayed for DON (Bluhm *et al.*, 2007). For infection assays with corn silks, inoculation with fungal culture blocks and disease symptom examination were performed as described by Seong *et al.* (2005). DON production *in vitro* was assayed with rice grain cultures (Bluhm *et al.*, 2007; Seo *et al.*, 1996) or liquid trichothecene biosynthesis (LTB) cultures in the presence of 5 mM $NH_4NO_3$ (Gardiner *et al.*, 2009a). For each strain, plant infection and DON production assays were repeated at least three times.

### Generation of the GFP-*FgATG8* transformants

To generate the GFP-*FgATG8* construct, the 1.5-kb *FgATG8* promoter region, eGFP and *FgATG8* were amplified with primers eGFPATG8/1F–eGFP-ATG8/1R, eGFPATG8/2F–eGFP-ATG8/2R and eGFPATG8/3F–eGFP-ATG8/3R, respectively (Table S3), and cloned into pFL2 by the yeast gap repair approach (Bruno *et al.*, 2004; Zhou *et al.*, 2011). The resulting GFP-*FgATG8* construct was confirmed by sequencing analysis and transformed into protoplasts of PH-1 and the *pkr* mutant P1. Transformants expressing the GFP-*FgATG8* construct were analysed by PCR and fluorescence microscopy.

### Cell wall, cell viability and glycogen staining

Conidia, germlings and hyphae of the wild-type strain PH-1 and the *pkr* mutant were stained with 10 μg/mL calcofluor white (CFW) and 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA), as described previously (Li *et al.*, 2015), to visualize cell wall and nuclei. Conidia were stained with 5 μg/mL propidium iodide (PI) (Sigma-Aldrich), as described previously (Palma-Guerrero *et al.*, 2009), for viability assays. Conidia were stained for glycogen with 60 mg/mL of KI and 10 mg/mL of $I_2$, as described previously (Qin *et al.*, 2015; Seong *et al.*, 2009). Septation, nuclear distribution, cell viability and glycogen accumulation were examined with an Olympus BX53 epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

### qRT-PCR analysis

Vegetative hyphae or germlings were isolated from YEPD cultures ($10^6$ conidia/100 mL) after shaking at 175 rpm at 25 °C for 12 h. RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with the Fermentas 1st cDNA synthesis kit (Hanover, MD, USA). The *F. graminearum* ubiquitin C-terminal hydrolase (*GzUBH*) gene was amplified with the primers GzUBHQF and GzUBHQR (Kim and Yun, 2011). Relative changes in the expression level of each gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *GzUBH* as the endogenous reference. For each gene, qRT-PCR data from three biological replicates were used to calculate the mean and standard deviation with two technical repeats each.

## ACKNOWLEDGEMENTS

We thank Qinhu Wang for assistance with phylogenetic tree analysis. We also thank Drs Huiquan Liu and Cong Jiang for fruitful discussions. This work was supported by grants from the Nature Science Foundation of China (No. 31571953), National Key Research and Development Foundation, Ministry of Science and Technology of China (2016YFD0300705), US Wheat and Barley Scab Initiative and Specialized Research Cultivation Fund for Excellent Young Scholars of Northwest Agricultural and Forestry University (NWSUAF).

## REFERENCES

**Alexander, N.J., Proctor, R.H. and McCormick, S.P.** (2009) Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. *Toxin Rev.* **28**, 198–215.

**Audenaert, K., Vanheule, A., Hofte, M. and Haesaert, G.** (2014) Deoxynivalenol: a major player in the multifaceted response of *Fusarium* to its environment. *Toxins*, **6**, 1–19.

**Bai, G.H. and Shaner, G.** (2004) Management and resistance in wheat and barley to Fusarium head blight. *Annu. Rev. Phytopathol.* **42**, 135–161.

**Bai, G.H., Desjardins, A.E. and Plattner, R.D.** (2002) Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia*, **153**, 91–98.

**Bluhm, B.H., Zhao, X., Flaherty, J.E., Xu, J.R. and Dunkle, L.D.** (2007) RAS2 regulates growth and pathogenesis in *Fusarium graminearum*. *Mol. Plant–Microbe Interact.* **20**, 627–636.

**Brown, D.W., Dyer, R.B., McCormick, S.P., Kendra, D.F. and Plattner, R.D.** (2004) Functional demarcation of the *Fusarium* core trichothecene gene cluster. *Fungal Genet. Biol.* **41**, 454–462.

**Bruno, K.S., Aramayo, R., Minke, P.F., Metzenberg, R.L. and Plamann, M.** (1996) Loss of growth polarity and mislocalization of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. *EMBO J.* **15**, 5772–5782.

**Bruno, K.S., Tenjo, F., Li, L., Hamer, J.E. and Xu, J.R.** (2004) Cellular localization and role of kinase activity of *PMK1* in *Magnaporthe grisea*. *Eukaryot. Cell*, **3**, 1525–1532.

**Choi, J., Jung, W.H. and Kronstad, J.W.** (2015) The cAMP/protein kinase A signaling pathway in pathogenic basidiomycete fungi: connections with iron homeostasis. *J. Microbiol.* **53**, 579–587.

**Cuomo, C.A., Guldener, U., Xu, J.R., Trail, F., Turgeon, B.G., Di Pietro, A., Walton, J.D., Ma, L.J., Baker, S.E., Rep, M., Adam, G., Antoniw, J., Baldwin, T., Calvo, S., Chang, Y.L., Decaprio, D., Gale, L.R., Gnerre, S., Goswami, R.S., Hammond-Kosack, K., Harris, L.J., Hilburn, K., Kennell, J.C., Kroken, S., Magnuson, J.K., Mannhaupt, G., Mauceli, E., Mewes, H.W., Mitterbauer, R., Muehlbauer, G., Munsterkotter, M., Nelson, D., O'Donnell, K., Ouellet, T., Qi, W., Quesneville, H., Roncero, M.I., Seong, K.Y., Tetko, I.V., Urban, M., Waalwijk, C., Ward, T.J., Yao, J., Birren, B.W. and Kistler, H.C.** (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science*, **317**, 1400–1402.

**Desjardins, A.E.** (2003) *Gibberella* from *A* (*venaceae*) to *Z* (*eae*). *Annu. Rev. Phytopathol.* **41**, 177–198.

**Ding, S.L., Mehrabi, R., Koten, C., Kang, Z.S., Wei, Y.D., Seong, K.Y., Kistler, H.C. and Xu, J.R.** (2009) Transducin beta-like gene *FTL1* is essential for pathogenesis in *Fusarium graminearum*. *Eukaryot. Cell*, **8**, 867–876.

**D'Souza, C.A., Alspaugh, J.A., Yue, C.L., Harashima, T., Cox, G.M., Perfect, J.R. and Heitman, J.** (2001) Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol. Cell. Biol.* **21**, 3179–3191.

**Fuller, K.K., Zhao, W., Askew, D.S. and Rhodes, J.C.** (2009) Deletion of the protein kinase A regulatory subunit leads to deregulation of mitochondrial activation and nuclear duplication in *Aspergillus fumigatus*. *Eukaryot. Cell*, **8**, 271–277.

**Gale, L.R., Ward, T.J., Balmas, V. and Kistler, H.C.** (2007) Population subdivision of *Fusarium graminearum* sensu stricto in the upper midwestern United States. *Phytopathology*, **97**, 1434–1439.

**Gardiner, D.M., Kazan, K. and Manners, J.M.** (2009a) Novel genes of *Fusarium graminearum* that negatively regulate deoxynivalenol production and virulence. *Mol. Plant–Microbe Interact.* **22**, 1588–1600.

**Gardiner, D.M., Kazan, K. and Manners, J.M.** (2009b) Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet. Biol.* **46**, 604–613.

**Gold, S., Duncan, G., Barrett, K. and Kronstad, J.** (1994) cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. *Genes Dev.* **8**, 2805–2816.

**Gold, S.E., Brogdon, S.M., Mayorga, M.E. and Kronstad, J.W.** (1997) The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize. *Plant Cell*, **9**, 1585–1594.

**Goswami, R.S. and Kistler, H.C.** (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* **5**, 515–525.

**Herberg, F.W., Doyle, M.L., Cox, S. and Taylor, S.S.** (1999) Dissection of the nucleotide and metal-phosphate binding sites in cAMP-dependent protein kinase. *Biochem. J.* **38**, 6352–6360.

**Hou, R., Jiang, C., Zheng, Q., Wang, C.F. and Xu, J.R.** (2015) The AreA transcription factor mediates the regulation of deoxynivalenol (DON) synthesis by ammonium and cyclic adenosine monophosphate (cAMP) signalling in *Fusarium graminearum*. *Mol. Plant Pathol.* **16**, 987–999.

**Hou, Z.M., Xue, C.Y., Peng, Y.L., Katan, T., Kistler, H.C. and Xu, J.R.** (2002) A mitogen-activated protein kinase gene (*MGV1*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol. Plant–Microbe Interact.* **15**, 1119–1127.

**Hu, S., Zhou, X.Y., Gu, X.Y., Cao, S.L., Wang, C.F. and Xu, J.R.** (2014) The cAMP–PKA pathway regulates growth, sexual and asexual differentiation, and pathogenesis in *Fusarium graminearum*. *Mol. Plant–Microbe Interact.* **27**, 557–566.

**Jiang, C., Zhang, S.J., Zhang, Q., Tao, Y., Wang, C.F. and Xu, J.R.** (2015) *FgSKN7* and *FgATF1* have overlapping functions in ascosporogenesis, pathogenesis and stress responses in *Fusarium graminearum*. *Environ. Microbiol.* **17**, 1245–1260.

**Jiang, C., Zhang, C.K., Wu, C.L., Sun, P.P., Hou, R., Liu, H.Q., Wang, C.F. and Xu, J.R.** (2016) *TRI6* and *TRI10* play different roles in the regulation of deoxynivalenol (DON) production by cAMP signalling in *Fusarium graminearum*. *Environ. Microbiol.* **18**, 3689–3701.

**Josefsen, L., Droce, A., Sondergaard, T.E., Sorensen, J.L., Bormann, J., Schafer, W., Giese, H. and Olsson, S.** (2012) Autophagy provides nutrients for nonassimilating fungal structures and is necessary for plant colonization but not for infection in the necrotrophic plant pathogen *Fusarium graminearum*. *Autophagy*, **8**, 326–337.

**Kennedy, E.J., Ghosh, G. and Pillus, L.** (2008) Identification of functionally distinct regions that mediate biological activity of the protein kinase a homolog Tpk2. *J. Biol. Chem.* **283**, 1084–1093.

**Kim, H.K. and Yun, S.H.** (2011) Evaluation of potential reference genes for quantitative RT-PCR analysis in *Fusarium graminearum* under different culture conditions. *Plant Pathol. J.* **27**, 301–309.

**Kim, J.E., Han, K.H., Jin, J.M., Kim, H., Kim, J.C., Yun, S.H. and Lee, Y.W.** (2005) Putative polyketide synthase and laccase genes for biosynthesis of aurofusarin in *Gibberella zeae*. *Appl. Environ. Microbiol.* **71**, 1701–1708.

**Kim, J.E., Jin, J.M., Kim, H., Kim, J.C., Yun, S.H. and Lee, Y.W.** (2006) GIP2, a putative transcription factor that regulates the aurofusarin biosynthetic gene cluster in *Gibberella zeae*. *Appl. Environ. Microbiol.* **72**, 1645–1652.

**Lee, N., D'Souza, C.A. and Kronstad, J.W.** (2003) Of smuts, blasts, mildews, and blights: cAMP signaling in phytopathogenic fungi. *Annu. Rev. Phytopathol.* **41**, 399–427.

**Li, C.H., Melesse, M., Zhang, S.J., Hao, C.F., Wang, C.F., Zhang, H.C., Hall, M.C. and Xu, J.R.** (2015) *FgCDC14* regulates cytokinesis, morphogenesis, and pathogenesis in *Fusarium graminearum*. *Mol. Microbiol.* **98**, 770–786.

**Li, G.T., Zhou, X.Y. and Xu, J.R.** (2012) Genetic control of infection-related development in *Magnaporthe oryzae*. *Curr. Opin. Microbiol.* **15**, 678–684.

**Livak, K.J. and Schmittgen, T.D.** (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods*, **25**, 402–408.

**Macheleidt, J., Mattern, D.J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V. and Brakhage, A.A.** (2016) Regulation and role of fungal secondary metabolites. *Annu. Rev. Genet.* **50**, 371–392.

**Mavrakis, M., Lippincott-Schwartz, J., Stratakis, C.A. and Bossis, I.** (2006) Depletion of type IA regulatory subunit (RIalpha) of protein kinase A (PKA) in mammalian cells and tissues activates mTOR and causes autophagic deficiency. *Hum. Mol. Genet.* **15**, 2962–2971.

**Menke, J., Weber, J., Broz, K. and Kistler, H.C.** (2013) Cellular development associated with induced mycotoxin synthesis in the filamentous fungus *Fusarium graminearum*. *PLoS One*, **8**, e63077.

**Merhej, J., Richard-Forget, F. and Barreau, C.** (2011) The pH regulatory factor *Pac1* regulates *Tri* gene expression and trichothecene production in *Fusarium graminearum*. *Fungal Genet. Biol.* **48**, 275–284.

**Nakatogawa, H., Ichimura, Y. and Ohsumi, Y.** (2007) Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell*, **130**, 165–178.

**Palma-Guerrero, J., Huang, I.C., Jansson, H.B., Salinas, J., Lopez-Llorca, L.V. and Read, N.D.** (2009) Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner. *Fungal Genet. Biol.* **46**, 585–594.

**Park, A.R., Fu, M., Shin, J.Y., Son, H. and Lee, Y.W.** (2016) The protein kinase A pathway regulates zearalenone production by modulating alternative *ZEB2* transcription. *J. Microbiol. Biotechnol.* **26**, 967–974.

**Qin, J., Wang, G.H., Jiang, C., Xu, J.R. and Wang, C.F.** (2015) Fgk3 glycogen synthase kinase is important for development, pathogenesis, and stress responses in *Fusarium graminearum*. *Sci. Rep.* **5**, 8504.

**Seo, J.A., Kim, J.C., Lee, D.H. and Lee, Y.W.** (1996) Variation in 8-ketotrichothecenes and zearalenone production by *Fusarium graminearum* isolates from corn and barley in Korea. *Mycopathologia*, **134**, 31–37.

**Seong, K., Hou, Z.M., Tracy, M., Kistler, H.C. and Xu, J.R.** (2005) Random insertional mutagenesis identifies genes associated with virulence in the wheat scab fungus *Fusarium graminearum*. *Phytopathology*, **95**, 744–750.

**Seong, K.Y., Pasquali, M., Zhou, X.Y., Song, J., Hilburn, K., McCormick, S., Dong, Y.H., Xu, J.R. and Kistler, H.C.** (2009) Global gene regulation by *Fusarium* transcription factors *Tri6* and *Tri10* reveals adaptations for toxin biosynthesis. *Mol. Microbiol.* **72**, 354–367.

**Staudohar, M., Bencina, M., van de Vondervoort, P.J.I., Panneman, H., Legisa, M., Visser, J. and Ruijter, G.J.G.** (2002) Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*. *Microbiology*, **148**, 2635–2645.

**Stephan, J.S., Yeh, Y.Y., Ramachandran, V., Deminoff, S.J. and Herman, P.K.** (2009) The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proc. Natl. Acad. Sci. USA*, **106**, 17 049–17 054.

**Takano, Y., Komeda, K., Kojima, K. and Okuno, T.** (2001) Proper regulation of cyclic AMP-dependent protein kinase is required for growth, conidiation, and appressorium function in the anthracnose fungus *Colletotrichum lagenarium*. *Mol. Plant–Microbe Interact.* **14**, 1149–1157.

**Trail, F.** (2009) For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. *Plant Physiol.* **149**, 103–110.

**Turra, D., Segorbe, D. and Di Pietro, A.** (2014) Protein kinases in plant-pathogenic fungi: conserved regulators of infection. *Annu. Rev. Phytopathol.* **52**, 267–288.

**Tzima, A., Paplomatas, E.J., Rauyaree, P. and Kang, S.** (2010) Roles of the catalytic subunit of cAMP-dependent protein kinase A in virulence and development of the soilborne plant pathogen *Verticillium dahliae*. *Fungal Genet. Biol.* **47**, 406–415.

**Voisey, C.R., Christensen, M.T., Johnson, L.J., Forester, N.T., Gagic, M., Bryan, G.T., Simpson, W.R., Fleetwood, D.J., Card, S.D., Koolaard, J.P., Maclean, P.H. and Johnson, R.D.** (2016) cAMP signaling regulates synchronised growth of symbiotic epichloe fungi with the host grass *Lolium perenne*. *Front. Plant Sci.* **7**, 1546.

**Wang, C., Zhang, S., Hou, R., Zhao, Z., Zheng, Q., Xu, Q., Zheng, D., Wang, G., Liu, H., Gao, X., Ma, J.W., Kistler, H.C., Kang, Z. and Xu, J.R.** (2011) Functional analysis of the kinome of the wheat scab fungus *Fusarium graminearum*. *PLoS Pathog.* **7**, e1002460.

**Xu, H.J., Wang, Y.J., Zhao, P.B., Zhang, Y.B., Xu, R.Y. and Li, D.C.** (2011) A cAMP-dependent protein kinase gene, *aapk1*, is required for mycelia growth, toxicity and pathogenicity of *Alternaria alternata* on tobacco. *J. Phytopathol.* **159**, 208–216.

**Yang, K.L., Qin, Q.P., Liu, Y.H., Zhang, L.M., Liang, L.L., Lan, H.H., Chen, C.H., You, Y.C., Zhang, F. and Wang, S.H.** (2016) Adenylate cyclase AcyA regulates development, aflatoxin biosynthesis and fungal virulence in *Aspergillus flavus*. *Front. Cell. Infect. Microbiol.* **6**, 190.

**Yin, T., Zhang, Q., Wang, J., Liu, H., Wang, C., Xu, J.R. and Jiang, C.** (2017) The cyclase-associated protein FgCap1 has both protein kinase A-dependent and -independent functions during deoxynivalenol production and plant infection in *Fusarium graminearum*. *Mol. Plant Pathol.* doi:10.1111/mpp.12540.

**Zhao, W., Panepinto, J.C., Fortwendel, J.R., Fox, L., Oliver, B.G., Askew, D.S. and Rhodes, J.C.** (2006) Deletion of the regulatory subunit of protein kinase a in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infect. Immun.* **74**, 4865–4874.

**Zheng, D.W., Zhang, S.J., Zhou, X.Y., Wang, C.F., Xiang, P., Zheng, Q. and Xu, J.R.** (2012) The FgHOG1 pathway regulates hyphal growth, stress responses, and plant infection in *Fusarium graminearum*. *PLoS One*, **7**, e49495.

**Zhou, X., Li, G. and Xu, J.R.** (2011) Efficient approaches for generating GFP fusion and epitope-tagging constructs in filamentous fungi. *Methods Mol. Biol.* **722**, 199–212.

**Zhou, X.Y., Heyer, C., Choi, Y.E., Mehrabi, R. and Xu, J.R.** (2010) The CID1 cyclin C-like gene is important for plant infection in *Fusarium graminearum*. *Fungal Genet. Biol.* **47**, 143–151.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1** Generation of the *pkr* deletion mutant. (A) The *PKR* locus and gene replacement construct. The *PKR* and *hph* genes are marked with open and filled arrows, respectively. E, *Eco*RI. (B) Southern blot analysis with the wild-type strain PH-1, *pkr* deletion mutants (P1 and P3) and false-positive transformant P2. All DNA samples were digested with *Eco*RI. The blots were hybridized with probe A (left) amplified with primers PKR/5F and PKR/6R, and probe B (right) amplified with primers H852 and H850.

**Fig. S2** The expression levels of genes related to aurofusarin biosynthesis assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA samples isolated from 12-h YEPD (1% yeast extract, 2% peptone, 2% glucose) cultures of PH-1 and the *pkr* mutant. The relative expression level of the *GIP1* (Fg02328), *GIP2* (Fg02320) and *PKS12* (Fg02324) genes in the wild-type was arbitrarily set to unity.

**Fig. S3** Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to assay the expression of *CPK1* and *CPK2* in the *pkr* mutant and suppressors. RNA samples were isolated from 12-h germlings harvested from YEPD (1% yeast extract, 2% peptone, 2% glucose) cultures of the wild-type PH-1 (WT), *pkr* mutant (*pkr*) and 12 suppressors (H1, H2, H3, H7, H39, H55, H61, H62, H63, H65, H66, H67). The expression level in PH-1 was arbitrarily set to unity.

**Table S1** Suppressor strains of the *pkr* mutant with various growth rates and conidiation.

**Table S2** Candidate genes sequenced in suppressor strains H3, H7 and H14.

**Table S3** Polymerase chain reaction (PCR) primers used in this study.