The cAMP-PKA Pathway Regulates Growth, Sexual and Asexual Differentiation, and Pathogenesis in *Fusarium graminearum*

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Like many other filamentous ascomycetes, *Fusarium graminearum* contains two genes named *CPK1* and *CPK2* that encode the catalytic subunits of cyclic AMP (cAMP)-dependent protein kinase A (PKA). To determine the role of cAMP signaling in pathogenesis and development in *F. graminearum*, we functionally characterized these two genes. In addition, we generated and characterized the *cpk1 cpk2* double and *fac1* adenylate cyclase gene deletion mutants. The *cpk1* mutant was significantly reduced in vegetative growth, conidiation, and deoxynivalenol production but it had increased tolerance to elevated temperatures. It was defective in the production of penetration branches on plant surfaces, colonization of wheat rachises, and spreading in flowering wheat heads. Deletion of *CPK1* had no effect on perithecial development but the *cpk1* mutant was defective in ascospore maturation and releasing. In contrast, the *cpk2* mutant had no detectable phenotypes, suggesting that *CPK2* contributes minimally to PKA activities in *F. graminearum*. Nevertheless, the *cpk1 cpk2* double mutant had more severe defects in vegetative growth and rarely produced morphologically abnormal conidia. The double mutant, unlike the *cpk1* or *cpk2* mutant, was nonpathogenic and failed to form perithecia on self-mating plates. Therefore, *CPK1* and *CPK2* must have overlapping functions in vegetative growth, differentiation, and plant infection in *F. graminearum*. The *fac1* mutant was also nonpathogenic and had growth defects similar to those of the *cpk1 cpk2* mutant. However, deletion of *FAC1* had no effect on conidium morphology. These results indicated that *CPK1* is the major PKA catalytic subunit gene and that the cAMP-PKA pathway plays critical roles in hyphal growth, conidiation, ascosporeogenesis, and plant infection in *F. graminearum*.

In many plant-pathogenic fungi, the cyclic AMP (cAMP)-dependent signaling pathway is one of the major signal transduction pathways that regulates various differentiation and infection processes (Brakhage and Liebmman 2005; Choi and Xu 2010; Lee et al. 2003; Lengeler et al. 2000; Li et al. 2012). The cAMP-dependent protein kinase A (PKA) is the major

downstream effector of the cAMP signaling pathway. In the absence of cAMP, a secondary messenger synthesized by adenylate cyclase, the PKA holoenzyme exists as an inactive heterotetramer composed of two regulatory (R) subunits and two catalytic (C) subunits. Binding of cAMP to R subunits leads to the activation of two monomeric C subunits, which can phosphorylate downstream target proteins and regulate a variety of physiological processes.

Filamentous fungi normally have two genes encoding the catalytic subunits of PKA and one of them often is responsible for most of the PKA activities (Banno et al. 2005; Lee et al. 2003; Ni et al. 2005; Schumacher et al. 2008). In a number of them, deletion of the major PKA catalytic subunit gene, such as *FoCPKA* of *Fusarium oxysporum*, *CPK1* of *F. verticillioides*, and *PKAC-1* of *Neurospora crassa*, results in reduced growth rate and conidiation, which is similar to deletion of the adenylate cyclase gene (Choi and Xu 2010; Kim et al. 2011; Shimizu and Keller 2001). However, deletion of the PKA catalytic subunit gene *CPKA* or adenylate cyclase gene *MAC1* in *Magnaporthe oryzae* had no significant effect on growth rate, although the *cpkA* mutant was delayed in appressorium formation and defective in appressorium turgor generation (Adachi and Hamer 1998; Choi and Dean 1997; Mitchell and Dean 1995). In *Botrytis cinerea*, conidiation was not impaired in the *Δbcpk1* mutant (Schumacher et al. 2008). Interestingly, conidiation is de-repressed in the *cr-1* adenylate cyclase mutant in *N. crassa* (Banno et al. 2005). In *Aspergillus nidulans*, the *pkaA* gene also negatively regulates asexual sporulation (Shimizu and Keller 2001).

In plant- and human-pathogenic fungi, cAMP signaling also is known to play a species-specific role in regulating sexual reproduction, pathogenesis, and other biological processes (Lee et al. 2003; Lengeler et al. 2000). For example, in *Ustilago maydis*, the adenylate cyclase mutant and the PKA catalytic subunit mutant grew similarly to the wild-type but were defective in pathogenesis on maize (Durrenberger et al. 1998). In *Cryptococcus neoformans*, the *cac1* adenylate cyclase mutant was defective in mating hypha production and failed to produce capsule and melanin (Alspaugh et al. 2002). Similar to the *cpkA* or *mac1* mutant in *M. oryzae*, mutants deleted of a PKA catalytic subunit gene or the adenylate cyclase gene in *Colletotrichum lagenarium* and *C. trifolii* were nonpathogenic and formed nonfunctional appressoria (Yamauchi et al. 2004; Yang and Dickman 1999). In *Alternaria alternata*, the *Δaapkl* mutant was reduced in *Alternaria alternata* toxin production and virulence (Xu et al. 2011). In *Verticillium dahlia*, PKA regulates ethylene biosynthesis, which is directly related to virulence (Tzima et al. 2010). In *B. cinerea*, the adenylate

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cyclase and PKA catalytic subunit genes are important for plant infection but deletion of these genes has no effect on the production of oxalic acid and botrydial, two important virulence factors (Schumacher et al. 2008).

F. graminearum is a major causal agent of wheat and barley head blight or scab worldwide (Bai and Shaner 2004). Fusarium head blight (FHB) poses a serious problem in wheat production by causing severe yield losses and contamination of infested kernels with harmful mycotoxins, including deoxynivalenol (DON) and zearalenone (Desjardins 2003; Goswami and Kistler 2004). In a previous study of systematic characterization of the *F. graminearum* kinome, we identified the mutants deleted of two PKA catalytic subunit genes named *CPK1* and *CPK2*. To better understand the cAMP-PKA pathway in *F. graminearum*, in this study, we further characterized the *cpk1* and *cpk2* mutants. In addition, we generated and characterized the *cpk1 cpk2* double and *fac1* adenylate cyclase gene deletion mutants. Whereas the *cpk2* mutant had no detectable phenotypes, the *cpk1* mutant had pleiotropic defects in growth, conidiation, DON production, and ascospore maturation. It was defective in the production of penetration branches on plant surfaces and colonization of wheat rachises. Although deletion of both PKA catalytic subunit genes is lethal in *Aspergillus nidulans* (Ni et al. 2005), the *cpk1 cpk2* double mutant in *F. graminearum* was viable, although it had more severe defects than the *cpk1* mutant and was sterile in self-crosses and non-pathogenic in infection assays. The *fac1* mutant had defects similar to those of the *cpk1 cpk2* double mutant in vegetative growth and pathogenesis but it still produced normal conidia. Overall, our data indicate that *CPK1* is the major PKA catalytic subunit in *F. graminearum* but *CPK1* and *CPK2* have overlapping functions. The cAMP-PKA pathway plays critical roles in hyphal growth, conidiation, ascosporeogenesis, and plant infection in *F. graminearum*.

RESULTS

CPK2 but not *CPK1* is dispensable for growth and conidiation.

The genome of *F. graminearum* contains two PKA catalytic subunit genes, FGSG_07251 and FGSG_08729, that were named *CPK1* and *CPK2*, respectively, in this study. Phylogenetic analysis revealed that these two genes are well conserved in other filamentous fungi (Supplementary Fig. S1). *CPK1* is orthologous to yeast *TPK2*, *CPKA* of *M. oryzae* (Mitchell and Dean 1995), and *adr1* of *U. maydis* (Durrenberger et al. 1998). The *cpk1* and *cpk2* mutants (Table 1) were generated in a previous study of the *F. graminearum* kinome (Wang et al. 2011). Whereas the *cpk2* mutant had no detectable phenotype, the

cpk1 mutant C1M-1 was significantly reduced in growth rate (Fig. 1A). Colonies formed by the *cpk1* mutant had limited aerial hyphal growth.

In comparison with the wild type, the *cpk1* mutant was reduced approximately 44% in conidiation in carboxy-methyl cellulose (CMC) cultures, which is not proportional to its ten-fold reduction in growth rate (Table 2). Microscopic examination revealed that the *cpk1* mutant tended to produce phialides and conidia earlier than the wild type. After incubation for 16 h, phialides and conidia were observed in CMC cultures of mutant C1M-1. Under the same conidiation, phialides were not produced by the wild-type strain PH-1 (Fig. 1B). It is likely that the *cpk1* mutant was defective in nutrient sensing and depressed in asexual reproduction, which may be related to its reduced vegetative growth. Although only data for the *cpk1* mutant C1M-1 and *cpk2* mutant C2M-1 were presented, mutants C1M-3 and C1M-6 and mutants C2M-8 and C2M-14 had phenotypes similar to C1M-1 and C2M-1, respectively.

For complementation assays, the wild-type *CPK1* allele was reintroduced into the *cpk1* mutant C1M-1. Defects in hyphal growth and other phenotypes described below were rescued in the resulting *cpk1/CPK1* transformant C1C-1 (Table 1). These results indicate that deletion of *CPK1* is directly responsible for the defects observed in the *cpk1* mutant and *CPK1* is important for normal vegetative growth and conidiation in *F. graminearum*.

Fig. 1. The *cpk1* mutant was defective in colony morphology and hyphal growth. **A**, Colonies formed by the wild type PH-1, *cpk1* mutant C1M-1, *cpk2* mutant C2M-1, and *cpk1/CPK1* complemented strain C1C-1 on potato dextrose agar plates. **B**, The formation of the conidiophores in the PH-1 and *cpk1* mutant C1M-1 in CMC medium after incubation for 16 h. Bar = 20 μm.

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

Strains	Brief description	Reference
PH-1	Wild-type	Cuomo et al. 2007
C1M-1	<i>cpk1</i> deletion mutant of PH-1	This study
C1M-3	<i>cpk1</i> deletion mutant of PH-1	This study
C1M-6	<i>cpk1</i> deletion mutant of PH-1	This study
C1C-1	<i>cpk1/CPK1</i> complemented transformant	This study
C2M-1	<i>cpk2</i> deletion mutant of PH-1	This study
C2M-7	<i>cpk2</i> deletion mutant of PH-1	This study
C2M-8	<i>cpk2</i> deletion mutant of PH-1	This study
C2M-14	<i>cpk2</i> deletion mutant of PH-1	This study
DM-1	<i>cpk1 cpk2</i> double mutant	This study
DM-8	<i>cpk1 cpk2</i> double mutant	This study
DM-9	<i>cpk1 cpk2</i> double mutant	This study
FM-1	<i>fac1</i> deletion mutant of PH-1	This study
FM-5	<i>fac1</i> deletion mutant of PH-1	This study
FM-7	<i>fac1</i> deletion mutant of PH-1	This study
FC-1	<i>fac1/FAC1</i> complemented transformant	This study

Elevated expression levels of genes related to conidiation and secondary metabolism in the *cpk1* mutant.

Several genes, including *HTF1*, *MCM1*, *FgSTUA*, and *FgCOS1*, are known to be important for conidiation in *F. graminearum* (Lysøe et al. 2011; Zheng et al. 2012b; Zhou et al. 2011; Zhou et al. 2009). In RNA samples isolated from 12-h complete medium (CM) cultures, the expression levels of *HTF1*, *MCM1*, *FgSTUA*, and *FgCOS1* were 57-, 12-, four-, and sevenfold, respectively, higher in the *cpk1* mutant than in PH-1 (Fig. 2). These results confirmed that *CPK1* plays a negative role in regulating conidiation under nutrient-rich conditions, which is consistent with the observation that the *cpk1* mutant was depressed in conidiation.

Because conidiation is often related to secondary metabolism (Calvo et al. 2002), we also assayed the expression levels of the *GIP1* laccase and *GIP2* C6 zinc finger transcription factor genes that are involved in aurofusarin biosynthesis (Kim et al. 2005, 2006). In comparison with PH-1, the *cpk1* mutant had 11- and 232-fold increase in the expression of the *GIP1* and *GIP2* genes, respectively. Therefore, *CPK1* also negatively regulates the expression of *GIP1*, *GIP2*, and possibly other aurofusarin biosynthesis genes.

In addition, DON is an important secondary metabolite in *F. graminearum*; therefore, we assayed the expression levels of the *TRI5*, *TRI6*, and *TRI12* genes in DON-producing cultures containing 5 mM arginine by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In comparison with that of the wild type, the expression level of *TRI5*, *TRI6*, or *TRI12* was reduced approximately fourfold in the *cpk1* mutant (Supplementary Fig. S2). In contrast, none of these three *TRI* genes had more than twofold reduction in the expression level in the *cpk2* mutant. In the *fac1* mutant, however, all of these three *TRI* genes were significantly reduced in expression and their transcripts were barely detectable.

Altered heat shock responses in the *cpk1* mutant.

Because cAMP signaling is known to be involved in heat tolerance, we assayed conidium germination and hyphal growth at 35°C. In the wild type, conidium germination was rarely observed, although conidium swelling and dead conidium compartments were observed after incubation in yeast extract peptone dextrose (YEPA) for 6 h or longer (Fig. 3A). Under the same conditions, conidium germination and germ tube growth were observed in the *cpk1* mutant (Fig. 3A). However, some conidium compartments and germ tubes became dead and empty by 24 h. Germ tube tips also tended to swell in the *cpk1* mutant. When assayed for growth on potato dextrose agar (PDA) plates, the wild-type strain formed compact colonies with limited growth at 35°C. The *cpk1* mutant had similar growth rate and colony morphology when cultured at 25 and 35°C (Fig. 3B). These results indicate that the *cpk1* mutant

was more tolerant to heat stress than the wild type for conidium germination and hyphal growth.

To further verify increased tolerance of the *cpk1* mutant to elevated temperatures, we assayed the expression of the *FgHSP30* (Fg01158), *FgHSP70* (Fg00838), and *FgGSY2* (Fg06822) genes. The orthologs of these genes are known to be related to heat shock responses in other fungi (Choi and Xu 2010; Lindquist and Craig 1988). When cultured at 35°C, the relative expression levels of *FgHSP30*, *FgHSP70*, and *FgGSY2* were 70-, 11-, and sevenfold higher, respectively, in the *cpk1* mutant than in PH-1. Therefore, deletion of *CPK1* resulted in elevated expression of heat stress response genes in *F. graminearum*.

CPK1 is required for ascospore germination and ascospore releasing.

On carrot agar plates, both *cpk1* and *cpk2* mutants were able to produce normal perithecia, indicating that neither of these two PKA catalytic subunit genes is essential for homothallic sexual reproduction in *F. graminearum*. However, in comparison with the wild type and the *cpk2* mutant, the *cpk1* mutant produced fewer perithecia in self-crosses (Fig. 4A). In addition, only a few perithecia formed by the *cpk1* mutant produced diminished cirrhi that appeared as small, pale drops on top of perithecia at 2 to 3 weeks postinduction for sexual reproduction. Under the same conditions, the wild-type, *cpk2* mutant,

Fig. 2. Expression levels of genes related to conidiation and aurofusarin biosynthesis assayed by quantitative reverse-transcriptase polymerase chain reaction. RNA samples isolated from 12-h CM cultures of PH-1 and the *cpk1* mutant. Relative expression level of the *FgSTUA* (Fg10129), *HTF1* (Fg16777), *FgCOS1* (Fg16816), *FgMCM1* (Fg08696), *GIP1* (Fg02328), and *GIP2* (Fg02320) genes in the wild type was arbitrarily set to 1.

Table 2. Phenotypes of the *cpk1*, *cpk2*, and *fac1* mutants in growth, conidiation, and plant infection

Strain	Growth rate (mm/day) ^{w,x}	Conidiation (×10 ⁵ conidia/ml) ^w	Disease inde ^{w,y}	DON (ppm) ^{w,z}	
				Wheat	Rice
PH-1 (wild type)	11.6 ± 0.3 a	12.5 ± 1.8 b	12.6 ± 0.5 a	1,523.6 ± 163.3 a	1,629.0 ± 294.4 a
C1M-1	1.6 ± 0.5 bc	7.0 ± 0.5 c	1.0 ± 0.0 b	147.01 ± 17.3 c	82.2 ± 54.4 b
C1C-1	11.4 ± 0.9 a	12.5 ± 1.3 b	12.4 ± 0.9 a	1,386.8 ± 247.6 ab	1,396.8 ± 293.0 a
C2M-1	11.3 ± 0.3 a	17.7 ± 2.4 a	13.0 ± 0.8 a	1,179.2 ± 102.4 b	1,466.5 ± 993.2 a
DM-1	0.8 ± 0.4 c	2.0 ± 0.5 d	0.6 ± 0.5 b	0.0 c	0.0 b
FM-1	2.0 ± 0.5 b	3.8 ± 0.8 d	0.5 ± 0.6 b	0.0 c	0.1 ± 0.2 b

^w The same letter following values indicates there was no significant difference; different letters indicate statistically significant differences ($P = 0.05$).

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

^y Diseased spikelets per wheat head examined 14 dpi. Mean and standard deviation were calculated with results from three independent experiments.

^z Flowering wheat heads were inoculated and infected wheat kernels were harvested for deoxynivalenol (DON) assays 14 days postinoculation (dpi). Autoclaved rice grains were inoculated and cultured for 21 days, then assayed for DON.

and complemented *cpk1/CPK1* strains produced extensive, yellowish cirrhi (Fig. 4A). These results indicate that the fertility of the *cpk1* mutant was reduced.

When examined under a compound microscope, perithecia produced by the *cpk1* mutant contained normal fascicles of asci, which had eight ascospores (Fig. 4B). However, many of the ascospores produced by the *cpk1* mutant strain C1M-1 were two-celled. Furthermore, mature ascospores of C1M-1 were often fragmented in the middle and each cell contained one or two nuclei visible by 4',6-diamido-2-phenylindole

staining (Fig. 4C). In the *cpk1/CPK1* transformant, ascospore morphology was normal. Therefore, although it is dispensable for ascus development and initial ascospore formation, *CPK1* is important for ascospore maturation and release in *F. graminearum*.

***CPK1* is important for virulence.**

In infection assays with flowering wheat heads, the wild type could cause typical head blight symptoms in the inoculated kernels and spread to other spikelets. On wheat heads inoculated with the *cpk1* mutant, only the inoculated floret developed scab symptoms 14 days postinoculation (dpi). It failed to spread to nearby spikelets (Fig. 5A). As a matter of fact, most of the time the *cpk1* mutant failed to spread from the inoculated kernel to the other two florets on the same spikelet. In contrast, the complemented strain C1C-1 and the *cpk2* mutant were as virulent as the wild type (Fig. 5A). The average disease index of the *cpk1* and *cpk2* mutants and PH-1 was 1.0, 13.0, and 12.6, respectively (Table 2).

Because DON is an important virulence factor, we assayed DON production in the *cpk1* and *cpk2* mutants. Whereas the *cpk2* mutant was normal, the *cpk1* mutant was significantly reduced in DON production in infested wheat kernels (Table 2). To confirm this observation, we assayed DON production in rice grain cultures as described (Bluhm et al. 2007; Seo et al. 1996). Similar to results with infested wheat grains, the *cpk1* mutant was significantly reduced in DON production in rice grain cultures but the *cpk1 cpk2* double mutant and the *fac1* mutant were blocked in DON biosynthesis (Table 2). DON production in these mutants is consistent with reduced expression of *TRI* genes, indicating that *CPK1* and *FAC1* play an important role in the regulation of *TRI* gene expression and DON biosynthesis.

Fig. 3. The *cpk1* mutant has increased tolerance to elevated temperatures. **A**, Conidia of PH-1 and the *cpk1* mutant were incubated in YEPD medium at 35°C. Germination and germ tube growth were examined after incubation for 6, 12, and 24 h. Bar = 20 μm. **B**, Colonies formed by PH-1 and the *cpk1* mutant C1M-1 on potato dextrose agar plates at 35°C. **C**, Quantitative reverse-transcriptase polymerase chain reaction assays for the relative expression of *FgHSP30* (Fg01158), *FgHSP70* (Fg00838), and *FgGSY2* (Fg06822). RNA samples were isolated from 12-h germlings harvested from CM cultures of PH-1 and C1M-1 that were incubated for 11 h at 25°C before switching to 35°C for 1 h. The expression level of each gene at 25°C for 12 h was arbitrarily set to 1.

Fig. 4. Ascospore maturation and release require *Cpk1* but not *Cpk2*. **A**, Mating cultures of PH-1, *cpk1* or *cpk2* mutant, and complemented *cpk1/CPK1* transformant were examined 2 weeks postinduction for sexual reproduction. The *cpk1* mutant produced fewer perithecia and rarely formed small, pale cirrhi. **B**, Asci and ascospores from perithecia formed by the PH-1 and the *cpk1* mutant. Bar = 20 μm. **C**, Ascospores of PH-1 and the *cpk1* mutant C1M-1 were stained with 4',6-diamido-2-phenylindole and Calcofluor white. Ascospores produced by the *cpk1* mutant were often two-celled and fragmented in the middle. Bar = 10 μm.

***CPK1* is important for plant colonization.**

To further determine the function of *CPK1* in plant infection, we examined the defects of the *cpk1* mutant in different infection processes. At 24 h postinoculation (hpi), direct penetration of wheat lemma epidermis by short, penetration branches (Wanjiru et al. 2002) was observed in the wild-type strain by scanning electron microscopy (SEM). The *cpk1* mutant was significantly reduced in the production of penetration branches and most of the germ tubes failed to penetrate underlying plant cells (Fig. 5B). By 48 hpi, extensive hyphal growth was observed in lemma tissues of wheat heads inoculated with PH-1. In contrast, fungal growth was restricted to the inner epidermal tissues in samples inoculated with the *cpk1* mutant (Fig. 5C). These results indicate that the *cpk1* mutant was reduced in the formation of penetration hyphae and delayed in the initial infection processes.

In addition to spreading to nearby spikelet is via the rachis, we also examined for fungal growth in the rachis of inoculated wheat heads. By 120 hpi, extensive fungal growth was observed in the vascular tissues of the rachis in wheat heads inoculated with PH-1. In wheat heads infected with the *cpk1* mutant, we failed to observe fungal hyphae in the rachis below or above the inoculated spikelet (Fig. 5D). Therefore, *CPK1* must be essential for spreading from inoculated florets to the rachis and colonization of vascular tissues in *F. graminearum*.

Deletion of *CPK1* or *CPK2* had only minor effects on the expression of the other *CPK* gene.

To determine whether deletion of *CPK1* affects the expression of *CPK2* or vice versa, RNA samples were isolated from germlings or vegetative hyphae of 3, 6, 12, 18, 24, and 48 h in CM cultures of PH-1 and the *cpk1* and *cpk2* mutants. The expression levels of *CPK1* and *CPK2* were assayed by qRT-PCR. In the wild type, the *CPK1* gene had the lowest expression level at 3 h and its expression increased over time (Fig. 6A). In comparison with 3-h germlings, *CPK1* expression increased more than twofold from 6 to 12 h (Fig. 6A). The *CPK2* gene had an expression profile similar to *CPK1* in the wild type but its expression level was lower than that of *CPK1* in germ tubes and hyphae, particularly at 6, 12, and 18 h (Fig. 6B).

In comparison with the wild type, the expression level of *CPK1* was increased in the *cpk2* mutant but the increase was less than twofold in all the time points assayed by qRT-PCR (Fig. 6C). In the *cpk1* mutant, the expression level of *CPK2* was increased slightly at 3 and 6 h (Fig. 6C) but reduced in other time points assayed. Therefore, deletion of *CPK1* or *CPK2* had only a minor effect (less than twofold) on the expression of the other PKA catalytic subunit gene in *F. graminearum*.

The *cpk1 cpk2* double mutant has severe defects in growth and conidiogenesis.

Based on the phenotypes of the *cpk1* and *cpk2* mutants, *CPK1* must encode the major catalytic subunit of PKA and *CPK2* plays only a minor role in *F. graminearum*. To further examine their functional relationship, we generated the *cpk1 cpk2* double mutant by deletion of *CPK1* in the *cpk2* mutant C2M-1 (Supplementary Fig. S3). The resulting double mutants (Table 1) were more significantly reduced in vegetative growth than the *cpk1* mutant (Fig. 7A; Table 2). Whereas the *cpk1* mutant still produced normal conidia and appeared to be de-repressed in conidiation in nutrient-rich conditions, the *cpk1 cpk2* double mutant rarely produced conidia (Table 2) that had abnormal morphology (Fig. 7B). In general, conidia produced by the *cpk1 cpk2* double mutant were smaller or shorter than the wild-type conidia. Many conidia produced by the double mutant had only one or two compartments (Fig. 7B).

The *cpk1 cpk2* double mutant

is nonpathogenic and sterile in self-crosses.

In infection assays with flowering wheat heads, the *cpk1 cpk2* mutant was nonpathogenic and failed to colonize the inoculated florets (Fig. 7C). The double mutant did not produce or produced barely detectable amount of DON (Table 2). The defects in growth and DON production may contribute to the loss of pathogenicity in the *cpk1 cpk2* mutant.

Because the *cpk1* mutant was defective in ascosporeogenesis, we also assayed the defect of the *cpk1 cpk2* mutant in sexual

Fig. 5. Defects of the *cpk1* mutant in plant infection. **A**, Flowering wheat heads were drop-inoculated with conidia from the wild type (PH-1), *cpk1* mutant (C1M-1), *cpk2* mutant (C2M-1), and complemented *cpk1/CPK1* transformant (C1C-1). Black dots mark the inoculated spikelets. Photographs were taken 14 days postinoculation. **B**, Lemma from the spikelets inoculated with PH-1 and C1M-1 were examined by SEM 24 h postinoculation (hpi). Bar = 5 μ m. The *cpk1* mutant was defective in the production of penetration branches. **C**, Colonization of lemma tissues by PH-1 and the *cpk1* mutant 48 hpi. Fungal hyphae were marked with arrows. Bar = 50 μ m. **D**, Rachises next to the inoculated spikelets were examined 120 hpi. Extensive hyphal growth in plant tissues (marked with arrows) were observed in samples inoculated with PH-1 but the *cpk1* mutant. Bar = 50 μ m.

reproduction. To our surprise, the *cpk1 cpk2* mutant was sterile in self-crosses. Although neither *CPK1* nor *CPK2* is essential for perithecium formation, the *cpk1 cpk2* mutant DM-1 failed to produce perithecia or protoperithecia on carrot agar cultures (Fig. 7D). The other two *cpk1 cpk2* double mutants (Table 1) had phenotypes similar to that of mutant DM-1. These results indicate that the double mutant displayed more severe defects in pathogenesis and sexual reproduction than the *cpk1* and *cpk2* mutants. Therefore, *CPK1* and *CPK2* must have overlapping functions in *F. graminearum* during plant infection and differentiation.

The *fac1* mutant differs from the *cpk1 cpk2* mutant in conidiation.

In addition to functionally characterizing the *CPK1* and *CPK2* genes, we also generated the knockout mutant of the *FAC1* (FGSG_01234) adenylate cyclase gene by the gene replacement approach. The *fac1* deletion mutant formed small, compact colonies with limited aerial hyphal growth (Fig. 8A).

In the wheat head and corn silk infection assays, the *fac1* mutant was nonpathogenic (Fig. 8B) and did not produce detectable amounts of DON (Table 2). These results indicate that the *fac1* mutant and *cpk1 cpk2* double mutant had similar defects in growth and pathogenesis. However, unlike the double mutant, the *fac1* mutant FM-1 produced morphologically normal conidia (Fig. 8C), although conidiation was significantly reduced (Table 2). The other two *fac1* mutants, FM-5 and FM-7 (Table 1), had defects similar to those of mutant FM-1.

DISCUSSION

The cAMP-PKA signaling pathway is involved in growth, development, nutrient sensing, mating, stress response, secondary metabolism, and pathogenicity in various fungi (Banno et al. 2005; Cervantes-Chavez et al. 2009; D'Souza and Heitman 2001; Lee et al. 2003; Lengeler et al. 2000; Liebmann et al. 2004). In many filamentous ascomycetes, including *A. nidulans*, *N. crassa*, and *M. oryzae*, there are two catalytic subunit genes

Fig. 6. Expression levels of the *CPK1* and *CPK2* genes assayed by quantitative reverse-transcriptase polymerase chain reaction. **A**, RNA samples were isolated from conidia of PH-1 cultured in YEPD medium for 3, 6, 12, 18, 24, and 48 h at 25°C. Relative expression levels of *CPK1* and *CPK2* were calculated with *GzUBH* gene as the internal control using the $2^{-\Delta\Delta CT}$ method (Kim and Yun 2011). The expression level of *CPK1* or *CPK2* at 3 h was arbitrarily set to 1. **B**, Relative expression levels of *CPK1* in comparison with *CPK2*. The expression level of *CPK2* was set to 1 for all the samples. **C**, Relative expression levels of *CPK1* and *CPK2* in the *cpk2* and *cpk1* mutants, respectively. Their expression level in PH-1 was arbitrarily set to 1.

and, usually, one of them is responsible for most of the PKA activity. The other one plays only a minor role or has no detectable functions (Lee et al. 2003; Ni et al. 2005; Schumacher et al. 2008). In *F. graminearum*, *CPK1* is orthologous to the major PKA catalytic subunit genes that have been characterized in other fungi and it plays a critical role in vegetative growth, asexual reproduction, sexual reproduction, and plant infection. In contrast, deletion of the minor PKA subunit gene *CPK2* had no detectable effects. It appears that the *CPK1* and *CPK2* genes do not regulate each other transcriptionally because deletion of one of them had no significant effect (more than twofold) on the expression of the other.

Interestingly, the *cpk1* mutant was reduced over 90% in growth rate but the conidiation level was only reduced by approximately 44%. Close examination indicated that the *cpk1* mutant formed abundant phialides at 16 h in CMC cultures and began to sporulate earlier than the wild type (Fig. 1). Suppression of conidiation in liquid cultures in the wild type is due to the presence of sufficient growth nutrients (Adams et al. 1998). Nutrients in CMC after 16 h of growth of the *cpk1* mutant were likely abundant, particularly because it had a reduced growth rate; therefore, conidiation should be suppressed, at least similarly to that of the wild type. Therefore, earlier conidiation observed in the *cpk1* mutant may be due to a defect in nutrient sensing, which leads to a de-repression of asexual reproduction. In poor nutrient medium, such as mung bean liquid medium, the *cpk1* mutant and PH-1 began to produce phialides and conidia at the same time (Supplementary Fig. S4). Conidiation also is de-repressed in the *cr-1* mutant of *N. crassa* (Banno et al. 2005) and the *pkaA* gene is known to negatively regulate asexual reproduction in *A. nidulans* (Ni et al. 2005). In *F. graminearum*, several transcription factor genes are known to play regulatory roles in conidiation. The expression of the *HTF1*, *MCM1*, *FgSTUA*, and *FgCOS1* genes was upregulated in the *cpk1* mutant, suggesting that deletion of *CPK1* may result in de-repression of conidiation in the presence of abundant nutrients. Nevertheless, as in many other fungal pathogens (Choi and Xu 2010; Tzima et al. 2010), deletion of the *CPKA* ortholog resulted in a reduction in conidiation in *F. graminearum* in 5-day-old CMC cultures. In addition, the *cpk1 cpk2* double mutant rarely produced conidia. Therefore, normal or efficient production of conidiation still requires proper regulation by the cAMP-PKA pathway.

Fig. 7. Defects of the *cpk1 cpk2* double mutant in growth, pathogenesis, sexual, and asexual reproduction. **A**, Colonies formed by PH-1 and the *cpk1 cpk2* double mutant DM-1 on 3-day-old potato dextrose agar cultures. **B**, Conidia of PH-1 and the *cpk1 cpk2* mutant in CMC cultures after 5 days. Bar = 20 μ m. **C**, Flowering wheat heads inoculated with PH-1 and the *cpk1 cpk2* mutant. Inoculation with H₂O was used as the negative control. **D**, Two-week-old carrot agar cultures of the *cpk1 cpk2* mutant.

Similar to its role in suppressing conidiation under nutrient-rich conditions, *CPK1* may also negatively regulate responses to heat stresses in *F. graminearum*. In comparison with the wild type, the *cpk1* mutant had increased heat tolerance and upregulated expression of heat shock protein genes *FgHSP30*, *FgHSP70*, and *FgGSY2* (Fig. 3C). In *F. verticillioides* and *N. crassa*, mutants defects in cAMP signaling also display increased tolerance to elevated temperatures (Supplementary Table S1) (Banno et al. 2005; Choi and Xu 2010).

As the primary inoculum, ascospores play a critical role in the disease cycle of *F. graminearum*. The *cpk1* mutant produced morphologically normal perithecia but it was reduced in perithecial formation and defective in cirrhi development in self-crosses. Most of the mature ascospores produced by the *cpk1* mutant were often two-celled and fragmented in the middle, suggesting that the cAMP-PKA pathway may be involved in ascosporeogenesis in *F. graminearum*. In several filamentous fungi, orthologs of Cpk1 are known to play an important role in sexual development (D'Souza and Heitman 2001; Lengeler et al. 2000). However, to our knowledge, the involvement of Cpk1 in ascosporeogenesis has not been reported. Fragmented ascospores suggest that the constriction and septum separating two daughter cells after the first mitosis in developing ascospores became complete in the *cpk1* mutant. Therefore, *CPK1* may be involved in regulating late stages of ascospore formation, particularly ascospore maturation. In *F. graminearum*, deletion of *MIDI1*, a mechanosensitive calcium ion channel gene, resulted in a reduction in forcible ascospore discharge and the production of abnormal, two-celled ascospores with constricted and fragile septa (Cavinder et al. 2011). In filamentous fungi, calcium signaling plays a major role in regulating

Fig. 8. Defects of the *fac1* mutant in growth and virulence. **A**, Three-day old potato dextrose agar cultures of the wild type (PH-1), *fac1* mutant (FM-1), and *fac1/FAC1* complemented transformant (FC-1). **B**, Wheat heads and corn silks inoculated with PH-1 and FM-1. **C**, Conidia of PH-1 and the *fac1* mutant in CMC cultures after 5 days. Bar = 20 μ m.

hyphal tip growth, branching, dimorphism, and the cell cycle (Bencina et al. 2005; Cavinder and Trail 2012). PKA-dependent phosphorylation regulates Ca^{2+} -channel activities in *A. niger* (Bencina et al. 2005). The defects of ascospores development and release in the *cpk1* mutant were similar to those of the *mid1* mutant, indicating that the cAMP-PKA pathway may cross-talk with Ca^{2+} signaling in *F. graminearum*.

As in many other plant-pathogenic fungi (Fuller and Rhodes 2012; Lee et al. 2003; Lengeler et al. 2000), *CPK1* and *FAC1* were found to be important for plant infection in *F. graminearum*. SEM examination revealed that deletion of the *CPK1* genes resulted in a significant reduction in the formation of penetration hyphae (Fig. 5B). In *M. oryzae* and other fungal pathogens, cAMP signaling is involved in surface recognition and plant-pathogen interaction (Lee et al. 2003; Ni et al. 2005; Schumacher et al. 2008). In *F. graminearum*, the cAMP-PKA pathway may also regulate the recognition and development of plant infection structures. In addition, the *cpk1* and *fac1* mutants were reduced or blocked in the production of DON (Table 2), which is one of the best characterized virulence factors in *F. graminearum* (Proctor et al. 1995). Defects in DON production may be directly responsible for the phenotypes of these mutants in spreading in flowering wheat heads and colonization of vascular tissues in the rachis. However, the *cpk1* and *fac1* mutants were reduced in growth rate, which may also contribute to their defects in plant infection.

In *M. oryzae*, deletion of the *CPKA* or *MAC1* gene does not affect growth rate but results in significant reduction in aerial hyphae (Adachi and Hamer 1998; Choi and Dean 1997; Mitchell and Dean 1995). However, in other filamentous fungi such as *F. verticillioides*, *N. crassa*, *A. nidulans*, and *F. oxysporum*, the adenylate cyclase mutant or PKA catalytic subunit mutant had significantly reduced growth rate (Choi and Xu 2010; Kim et al. 2011). These results indicated that the role of cAMP signaling in vegetative growth is different among diverse fungal species. In *F. verticillioides*, the *fac1* mutant grew more slowly than the *cpk1* mutant. However, in this study, we found that the *cpk1 cpk2* double mutant had more severe defects in conidiation and virulence than the *fac1* mutant. Due to the conserved nature of the cAMP-PKA pathway, it is unlikely that the PKA catalytic subunit genes are activated by other mechanisms. The catalytic subunits of PKA in *F. graminearum* may have residential activities without the binding of cAMP to the regulatory subunit of PKA. PKA may maintain a basal level activity even in the absence of *FAC1* and cAMP in *F. graminearum* (Ni et al. 2005).

Although deletion of both catalytic subunits of PKA (*Adr1* and *Uka1*) was not lethal in the basidiomycete pathogen *U. maydis* (Durrenberger et al. 1998), the *tpk1 tpk2 tpk3* triple mutant was not viable in *Saccharomyces cerevisiae* (Toda et al. 1987). In *A. nidulans*, the overexpression of *pkaB* can rescue the growth and spore germination defects caused by $\Delta pkaA$, and deletion of both *pkaB* and *pkaA* is lethal, indicating that PkaA and PkaB have overlapping functions (Ni et al. 2005). It has been generally assumed that the *cpk1 cpk2* double mutant was nonviable in *M. oryzae* (Choi and Xu 2010). In this study, we found that the *cpk1 cpk2* mutant was viable, which is the first report of mutants with both PKA catalytic subunit genes deleted in filamentous ascomycetes. In comparison with the *cpk1* mutant, the double mutant had more severe defects in growth, sexual reproduction, and conidiation. It rarely produced conidia (Fig. 7B) and was nonpathogenic and sterile in self-crosses. In *U. maydis*, *Adr1* is the major catalytic subunit of PKA but the *adr1 uka1* double mutant also had more severe defects than the *adr1* (Durrenberger et al. 1998). In *F. graminearum* and possibly other filamentous ascomycetes, the two catalytic subunits of PKA likely have overlapping functions in

regulating hyphal growth, sexual and asexual reproduction, and plant infection. Therefore, it will be important to characterize substrate specificities of *CPK1* and *CPK2* and their contributions to PKA activities in different developmental and infection processes.

MATERIALS AND METHODS

Strains and culture conditions.

The wild-type strain PH-1 (Cuomo et al. 2007) and mutants of *F. graminearum* generated in this study (Table 1) were routinely cultured at 25°C on PDA agar plates. Growth rate on PDA plates and conidiation in CMC medium were assayed as described previously (Ding et al. 2009; Zhou et al. 2010). For DNA and RNA extraction, vegetative hyphae or germlings were harvested from liquid YEPD (1% yeast extract, 2% peptone, and 2% glucose). Protoplast preparation and fungal transformation were performed as described (Hou et al. 2002; Proctor et al. 1995). For transformation, hygromycin B (CalBiochem, La Jolla, CA, U.S.A.) and geneticin (Sigma-Aldrich, St. Louis) were added to the final concentration at 300 and 350 µg/ml, respectively.

Generation of the *cpk1 cpk2* and *fac1* mutants.

The *cpk1* and *cpk2* mutants were generated in a previous study by the split-marker approach (Wang et al. 2011). The same method was used to generate the *fac1* deletion mutant by transforming the overlapping PCR products into the protoplasts of PH-1. For generating the *cpk1 cpk2* double mutants, the *CPK1* gene-replacement construct generated with the neomycin resistance gene (*NEO^R*) was transformed into the *cpk2* mutant C2M-1. The putative knockout mutants were identified by PCR and further confirmed by Southern blot hybridizations.

For complementation assays, the entire *CPK1* gene was amplified with primers CM-CPK1/F and CM-CPK1/R, digested with *SacI* and *EcoRI*, and cloned into the *NEO^R* vector pHZ100 (Bluhm et al. 2007). The resulting construct, pCPK1, was transformed into protoplasts of the *cpk1* mutant C1M-1. The same method was used to generate the *fac1/FAC1* complemented transformants.

Sexual reproduction assays.

To induce sexual reproduction, aerial hyphae of 7-day-old carrot agar cultures were pressed down with sterile 0.1% Tween 20 and incubated at 25°C, as described (Wang et al. 2011; Zheng et al. 2013). Perithecia formation, cirrhi production, and ascospores were examined 2 to 3 weeks after induction. Each experiment was repeated independently three times.

Plant infection and DON production assays.

Conidia of PH-1 and mutant strains were harvested from 5-day-old CMC cultures and resuspended to 10^6 spores/ml, as described (Hou et al. 2002). Flowering wheat heads of 'Norm' were inoculated with 10 µl of conidium suspensions at the fifth spikelet from the base of the inflorescence, as described (Ding et al. 2009; Gale et al. 2007). Spikelets with typical head blight symptoms were examined 14 dpi and infected wheat kernels were harvested for DON assays, as described (Bluhm et al. 2007). For infection assays with mature corn plants of 'Pioneer 2375', sterile toothpicks were soaked in conidium suspensions before being pushed into the stalks as described (Choi and Xu 2010). Infection assays with corn silks were conducted as described (Seong et al. 2005). DON production in rice grain cultures was assayed as described (Bluhm et al. 2007; Seo et al. 1996). All the infection and DON production assays were repeated at least three times.

Microscopic observations.

Glumes and lemmas were collected from inoculated spikelets and fixed with 4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) overnight at 4°C. After dehydration in a series of acetone (30, 50, 70, 80, 90, and 100% [vol/vol]), the samples were mounted on stubs, sputter coated with gold-palladium, and examined with a JEOL 6360 scanning electron microscope (Jeol Ltd., Tokyo). For light microscope examination, infected wheat lemma and rachis tissues were fixed, dehydrated, and embedded in Spurr resin, as described (Kang et al. 2008; Zheng et al. 2012a). Thick sections (1 µm) were stained with 0.5% (wt/vol) toluidine blue and examined with an Olympus BX-53 microscope (Olympus, Tokyo). At least three independent biological replicates were examined for the wild-type and mutant strains.

qRT-PCR analysis.

Vegetative hyphae or germlings were isolated from CM cultures of PH-1 and the *cpk1* or *cpk2* mutant (10⁶ conidia per 100 ml) after shaking at 175 rpm for 3, 6, 12, 18, 24, and 48 h at 25°C. DON production and *TRI* gene expression in the presence of 5 mM arginine were assayed as described (Gardiner et al. 2009). RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). First-strand cDNA was synthesized with the Fermentas First cDNA synthesis kit (Hanover, MD, U.S.A.) following the instructions provided by the manufacturer. The *F. graminearum* ubiquitin C-terminal hydrolase (*GzUBH*) gene was amplified with primers GzUBHQF and GzUBHQR (Kim and Yun 2011). Relative changes in the expression level of each gene were calculated by the 2^{-ΔΔC_t} 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.

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