**Summary**

The biosynthesis of mycotoxin deoxynivalenol (DON) in *Fusarium graminearum* is regulated by two pathway-specific transcription factors Tri6 and Tri10 and affected by various host and environmental factors. In this study, we showed that cyclic adenosine monophosphate (cAMP) treatment induced DON production by stimulating TRI gene expression and DON-associated cellular differentiation in *F. graminearum*. Interestingly, exogenous cAMP had no effects on the tri6 mutant but partially recovered the defect of tri10 mutant in DON biosynthesis. Although the two cAMP phosphodiesterase genes PDE1 and PDE2 had overlapping functions in vegetative growth, conidiation, sexual reproduction and plant infection, deletion of PDE2 but not PDE1 activated intracellular PKA activities and increased DON production. Whereas the tri6 pde2 mutant failed to produce DON, the tri10 pde2 double mutant produced a significantly higher level of DON than the tri10 mutant. Cellular differentiation associated with DON production was stimulated by exogenous cAMP or deletion of PDE2 in both tri10 and tri6 mutants. These data indicate that TRI6 is essential for the regulation of DON biosynthesis by cAMP signalling but elevated PKA activities could partially bypass the requirement of TRI10 for TRI gene-expression and DON production, and Pde2 is the major cAMP phosphodiesterase to negatively regulate DON biosynthesis in *F. graminearum*.

**Introduction**

Fusarium head blight (FHB) caused by *Fusarium graminearum* is a major threat to global wheat production (Goswami and Kistler, 2004). In addition to severe yield losses and grain quality reduction, *F. graminearum* produces trichothecene mycotoxin deoxynivalenol (DON) and other toxic secondary metabolites in infested grains. DON is a potent inhibitor of protein synthesis in eukaryotic organisms. It is harmful to human and animal health and plays a critical role in plant infection (De Walle et al., 2010; Audenaert et al., 2014).

All the TRI genes involved in trichothecene biosynthesis have been identified in *F. graminearum* and *Fusarium sporotrichioides* (Brown et al., 2004; Alexander et al., 2009). Except TRI1, TRI16 and TRI101, the other TRI genes are in the main TRI1 gene cluster. Among them, TRI6 and TRI10 are two major transcriptional regulators of TRI gene expression (Seong et al., 2009). The binding site of Tri6 in the promoters of TRI genes have been identified and characterized in previous studies (Seong et al., 2009; Nasmith et al., 2011). Although the exact function of Tri10 is not clear, deletion of TRI10 in *F. graminearum* also significantly reduces TRI1 gene expression (Seong et al., 2009). Nevertheless, it is not clear how the Tri6 and Tri10 transcription factors are activated to regulate the expression of other TRI genes. The functional relationship between Tri6 and Tri10 also is not clear. Interestingly, cyclic adenosine monophosphate (cAMP) signalling and all three MAP kinase pathways have been shown to be important for DON biosynthesis in *F. graminearum* (Hou et al., 2002; Jenziemionka and Schafer, 2005; Zheng et al., 2012; Hu et al., 2014). It is likely that some of these key signalling pathways are involved in transducing extracellular signals to regulate TRI gene expression via Tri6 and Tri10 activation. Various environmental factors, such as nitrogen sources, pH and ROS, are known to affect DON biosynthesis in *F. graminearum* (Merhej et al., 2011; Hou et al., 2015; Jiang et al., 2015).
As a key secondary messenger, cAMP synthesized by adenylate cyclase plays a central role in the transduction of environmental stimuli to its downstream target cAMP protein kinase A (PKA). In the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*, cAMP signalling regulates nutrient sensing, spore germination and mating processes. The adenylate cyclase gene and genes encoding regulatory and catalytic subunits of PKA have been functionally characterized in a number of fungal pathogens (Kronstad et al., 1998; Borges-Walmsley and Walmsley, 2000; Lee et al., 2003; Choi and Xu, 2010). In addition to its conserved roles in pathogenesis, the cAMP-PKA pathway is important for various growth and developmental processes in different fungi, such as dimorphic switch and mating in *Ustilago maydis* and surface recognition in *Magnaporthe oryzae* (Durrenberger et al., 1998; Ramanujam and Naqvi, 2010). In addition, cAMP signalling has been implicated in regulating secondary metabolisms in several fungi, including *F. graminearum* and *Aspergillus* species (Brakhage and Liebmann, 2005; Hu et al., 2014). In *F. graminearum*, functional characterizations of the *FAC1*, *CPK1* and *CPK2* genes have shown that the cAMP-PKA pathway plays a role in morphogenetic switch from vegetative growth to pathogenic lifestyle, DON production, and sexual reproduction (Borman et al., 2014; Hu et al., 2014).

Like in other organisms, cAMP is hydrolyzed to 5‘-AMP by phosphodiesterases (PDEs) (Moorthy et al., 2011). In *S. cerevisiae*, whereas the high affinity phosphodiesterase Pde2 controls the basal level of cAMP and protects the cells from extracellular cAMP, the low affinity phosphodiesterase Pde1 regulates cAMP level induced by glucose stimulation or acidification (Ma et al., 1999). Deletion of *PDE2* but not *PDE1* led to an increased cell size, abnormal cell wall morphology and increased sensitivities to osmotic and oxidative stresses (Park et al., 2005). However, *S. pombe* has only one phosphodiesterase that functions to modulate intracellular cAMP level (Matwij et al., 1993). Like the budding yeast, most filamentous fungal pathogens have two *PDE* genes. In *M. oryzae*, deletion of *PDEH*, an ortholog of yeast *PDE2*, results in an elevated intracellular cAMP level and defects in appressorium development but deletion of *PDEL* has no obvious phenotypes (Ramanujam and Naqvi, 2010; Zhang et al., 2011). In *Botrytis cinerea*, the *pde2* deletion mutant is defective in vegetative growth, differentiation and virulence although it has a reduced intracellular cAMP level (Harren et al., 2013). In human pathogen *Candida albicans*, *PDE2* deletion mutant also has an increased intracellular cAMP level and it is defective in nutrient sensing, filamentation and cell wall and membrane integrity (Jung et al., 2005). Nevertheless, Pde1 but not Pde2 regulates the basal CAMP level in *Cryptococcus neoformans* (Hicks et al., 2005).

To further characterize the relationship between intracellular cAMP and DON biosynthesis, in this study, we determined the effects of exogenous cAMP on DON production and cellular differentiation associated with DON production in the wild type and *tri6* and *tri10* deletion mutants. Interestingly, we found that cAMP treatment could partially suppress the defects of *tri10* but not *tri6* mutant in DON biosynthesis. We then functionally characterized the two phosphodiesterase genes, *PDE1* and *PDE2*, in *F. graminearum*. Although they have overlapping functions in growth, sexual and asexual reproduction and plant infection, *PDE2* plays a major role in negatively regulating DON production. Deletion of *PDE2* also could partially recovered DON production of *tri10* but not *tri6* mutant. Our results showed that the cAMP-PKA pathway interacts differently with *TRI6* and *TRI10*, and two *PDE* genes have distinct and overlapping functions in regulating growth, development, infection and secondary metabolism in *F. graminearum*.

**Results**

*Exogenous cAMP increases DON production in F. graminearum*

To determine the effect of cAMP on trichothecene synthesis, the wild-type strain PH-1 (Table 1) was treated with 1, 2 or 4 mM cAMP in Liquid trichothecene biosynthesis (LTB) medium (Gardiner et al., 2009) and assayed for DON production after incubation for 7 days (Fig. 1A). Although treatment with 1 mM cAMP had only a slight effect, DON production was increased approximately 40-folds in cultures treated with 4 mM cAMP (Fig. 1A). As the negative control, exogenous cAMP had no effect on the *cpk1 cpk2* mutant (Hu et al., 2014) for DON production (Supporting Information Fig. S1). When assayed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), all the *TRI* genes assayed had increased expression levels in cultures treated with 4 mM cAMP (Fig. 1B). The expression level of *TRI5* that encodes the key trichodiene synthase for DON biosynthesis was increased approximately 45-folds.

Because intracellular cAMP is degraded by cAMP phosphodiesterases (Moorthy et al., 2011), we also assayed the effects of phosphodiesterase inhibitors on DON production in PH-1. In the presence of 5 mM IBMX, DON production in 7-day-old LTB cultures was increased 8.5-fold in comparison with that of normal PH-1 cultures (Fig. 1C). Caffeine treatment also significantly increased DON production in *F. graminearum* (Fig. 1C).

*Exogenous cAMP increases DON production in F. graminearum*
compartments (Jonkers et al., 2012; Menke et al., 2013). In PH-1 cultures treated with 4 mM cAMP, more abundant bulbous structures were observed than the untreated control after incubation for 3 days (Fig. 2A). In fact, hyphal bulbous structures became visible in cAMP-treated cultures as early as in two days (Fig. 2A). In the wild type, hyphal bulbous structures were observed only after incubation more than 2 days.

We then assayed DON production in LTB cultures of PH-1 treated with 4 mM cAMP. Production of DON was only detectable in 5-day-old cultures. After incubation for 5 days, DON production in cAMP-treated cultures was 11.3-times higher than untreated cultures (Fig. 2B). In the untreated controls, DON was only detectable in 5-day-old cultures. In PH-1 cultures treated with 4 mM cAMP, more abundant bulbous structures were observed than the untreated control after incubation for 3 days (Fig. 2A). In fact, hyphal bulbous structures became visible in cAMP-treated cultures as early as in two days (Fig. 2A). In the wild type, hyphal bulbous structures were observed only after incubation more than 2 days.

We then assayed DON production in LTB cultures of PH-1 treated with 4 mM cAMP. Production of DON was only detectable in 5-day-old cultures. After incubation for 5 days, DON production in cAMP-treated cultures was 11.3-times higher than untreated cultures (Fig. 2B). These results indicate that cAMP treatment stimulated cellular differentiation and DON production in juvenile cultures.

Exogenous cAMP partially bypasses the function of TRI10 in DON regulation

In F. graminearum, TRI6 and TRI10 are two pathway-specific transcriptional regulators of TRI gene expression and DON production. To determine the effects of cAMP treatment on TRI gene regulation, we treated arginine-containing LTB cultures of the tri6 and tri10 mutants (Table 1) with 4 mM cAMP. Although exogenous cAMP had no effects on the tri6 mutant, DON production was partially recovered in the tri10 mutant (Fig. 3A). When assayed by qRT-PCR, the expression level of TRI5 was increased threefold in the tri10 mutant in cultures treated with cAMP in comparison with untreated controls (Fig. 3B). In the tri6 mutant, the expression level of TRI5 was not affected by exogenous cAMP (Fig. 3B). These results indicate that cAMP treatment could partially suppress the effects of TRI10 deletion on TRI gene expression. However, TRI6 is essential for the regulation of DON biosynthesis by the cAMP-PKA pathway.

Deletion of PDE1 or PDE2 has no significant effects on conidiation, sexual reproduction and plant infection

Like other eukaryotes, the intracellular cAMP level is regulated by adenylyl synthase and cAMP phosphodiesterase (PDE) in fungi. The F. graminearum genome contains two putative phosphodiesterase genes, FGSG_06633 and FGSG_06914 that are named PDE1 and PDE2 respectively, in this study. PDE1 encodes a 502-aa polypeptide that has a conserved class II PDE consensus and is orthologous to the low-affinity phosphodiesterase Pde1 of S. cerevisiae and M. oryzae (Ma et al., 1999; Zhang et al., 2011). In contrast, PDE2 encodes an 879-aa protein with a conserved class I PDE consensus and it is orthologous to high-affinity phosphodiesterase Pde2 of the budding yeast and M. oryzae (Park et al., 2005; Ramanujam and Naqvi, 2010) (Supporting Information Fig. S2).

To determine the functions of these two PDE genes and their roles in cAMP signalling in F. graminearum, we generated the pde1 and pde2 deletion mutants in the wide-type strain PH-1 by the split-marker approach (Catlett et al., 2003) (Table 1). In comparison with PH-1, the pde1 mutant had no obvious defects in vegetative growth and colony morphology (Fig. 4A). The pde2 mutant formed compact colonies that was slightly reduced in growth rate but the reduction was not statistically significant (Fig. 4A; Table 2). Nevertheless, both pde1 and pde2 mutants were normal in sexual reproduction (Fig. 4B), conidiation (Table 2), and plant infection (Fig. 4C). These results indicate that, unlike the importance of PDE2 in asexual development and pathogenicity in M. oryzae and B. cinerea (Ramanujam

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1</td>
<td>Wild-type</td>
<td>(Cuomo et al., 2007)</td>
</tr>
<tr>
<td>DM-1</td>
<td>tri6 deletion mutant of PH-1</td>
<td>(Seong et al., 2009)</td>
</tr>
<tr>
<td>L1</td>
<td>pde1 deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>L2</td>
<td>pde1 deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>H2</td>
<td>pde2 deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>H3</td>
<td>pde2 deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
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<td>pde1 pde2 double mutant</td>
<td>This study</td>
</tr>
<tr>
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<td>pde1 pde2 double mutant</td>
<td>This study</td>
</tr>
<tr>
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<td>tri6 pde2 double mutant</td>
<td>This study</td>
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<tr>
<td>DH62</td>
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</tr>
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</tr>
<tr>
<td>DH103</td>
<td>tri10 pde2 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>HC1</td>
<td>PDE2-GFP complemented transformant of H3</td>
<td>This study</td>
</tr>
<tr>
<td>LC3</td>
<td>PDE1-RFP complemented transformant of L2</td>
<td>This study</td>
</tr>
</tbody>
</table>

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and Naqvi, 2010; Harren et al., 2013), deletion of either PDE2 or PDE1 had no significant effects on virulence and sexual reproduction in *F. graminearum*.

Deletion of PDE2 significantly increases DON production and TRI gene expression

When assayed with LTB cultures, the *pde1* mutant was only slightly increased in DON production in comparison with PH-1. However, DON production was increased over 91.3-fold in the *pde2* mutant (Fig. 5A). Furthermore, the expression level of TRI5 was increased 84.5-fold in the *pde2* mutant in comparison with PH-1 (Fig. 5B). These data indicate that PDE2 but not PDE1 plays a negative role in the regulation of DON biosynthesis. We also assayed DON production with rice grain cultures and inoculated wheat kernels that developed FHB symptoms by 14 dpi. Similar to results obtained with LTB cultures, DON production was 9.8- and 9.4-folds higher in rice grain cultures of *pde2* mutant than those of PH-1 or *pde1* mutant (Table 2). Although it was reduced in virulence, the *pde2* mutant produced a higher concentration of DON in the diseased wheat kernels than the wild type and *pde1* mutant (Table 2).

![Fig. 1. Effects of exogenous cAMP on DON production and TRI gene expression in the wild-type strain. A. DON production in LTB cultures treated with 0 (CK), 1, 2 and 4 mM cAMP after incubation for 7 days. B. TRI gene expression assayed by qRT-PCR with RNA samples isolated from cultures incubated in the presence of 0 or 4 mM cAMP for 3 days. The relative expression level of each gene in cultures without exogenous cAMP was arbitrarily set to 1. C. 7-day-old LTB cultures with or without 10 mg/ml caffeine or 5 mM IBMX. Mean and standard error were calculated from three independent biological replicates.](image1)

![Fig. 2. Stimulation of cellular differentiation associated with DON biosynthesis by cAMP.](image2)

1. LTB cultures of the wild-type strain PH-1 treated with or without 4 mM cAMP were examined for bulbous structures (marked with arrows) after incubation for 1, 2 and 3 days. Bar = 10 μm.
2. Assays for DON production in LTB cultures treated with 0 (black circles) or 4 mM (white circles) cAMP after incubation for 1, 3 and 5 days.

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When assayed for their expressions in YEPD and LTB cultures, the expression of \textit{PDE2} but not \textit{PDE1} was downregulated under DON-producing conditions (Fig. 5C), which is consistent with a negative role of \textit{PDE2} in DON regulation. After being incubated in LTB cultures for 3 days, bulbous structures were rarely observed in \textit{PH-1} and the \textit{pde1} mutant. Under the same conditions, abundant bulbous hyphal structures were observed in the \textit{pde2} mutant (Fig. 5D), which is consistent with increased DON production.

For complementation assays, we generated the \textit{PDE1-RFP} and \textit{PDE2-GFP} constructs under the control of their native promoters and transformed them into the \textit{pde1} and \textit{pde2} mutants respectively. In the resulting \textit{pde1/PDE1-RFP} or \textit{pde2/PDE2-GFP} transformants, no or only faint GFP signals were observed. Like the \textit{pde1} mutant, the \textit{pde1/PDE1-RFP} transformant had the wild-type growth rate and colony morphology. However, the \textit{pde2/PDE2-GFP} transformant was normal in colony growth and DON production (Supporting Information Fig. S3), indicating that deletion of \textit{PDE2} was responsible for the mutant phenotypes observed.

\textbf{PDE1 and PDE2 have overlapping functions in growth, development and pathogenesis}

We then generated the \textit{pde1 pde2} double mutant by deletion of \textit{PDE2} in the \textit{pde1} mutant (Table 1). The resulting \textit{pde1 pde2} double mutant had a significant reduction in growth rate than the \textit{pde2} single mutant (Table 2) and produced compact colonies with limited aerial hyphae (Fig. 4A). In the presence of 2 mM cAMP, the growth rate of \textit{pde2} mutant was significantly reduced in comparison with that of the wide-type \textit{PH-1} or \textit{pde1} mutant. However, growth of the double mutant was completely blocked.
Supporting Information Fig. S4), indicating that exogenous cAMP may be inhibitory to fungal growth when the cAMP degradation system is abolished.

Although the pde1 and pde2 mutants were normal in perithecia development and ascospore release, the pde1 pde2 double mutant was sterile and failed to develop perithecia 2 weeks post-fertilization (Fig. 4B) or after longer incubation. The double mutant also was significantly reduced in conidiation and virulence (Table 2). It rarely produced conidia and conidia of the double mutant tended to have abnormal morphology (Supporting Information Fig. S5). In infection assays with wheat heads, the pde1 pde2 double mutant has a disease index of less than 1 (Table 2) and caused FHB symptoms only on the inoculated kernels (Fig. 4C), indicating that it was defective in spreading from the inoculation site to nearby spikelets. Therefore, PDE1 and PDE2 must have overlapping functions in hyphal growth, conidiation, sexual development and plant infection in F. graminearum.

We also assayed DON production with the pde1 pde2 double mutant. In comparison with the pde2 mutant, the pde1 pde2 double mutant was increased in DON production in rice grain cultures and diseased wheat kernels (Table 2). These results suggest that, although Pde2 is the

Fig. 5. PDE2 negatively regulates DON production and TRI gene expression.
A. DON production in 7-day-old LTB cultures of the wide type PH-1, pde1 mutant, and pde2 mutant.
B. TRI5 expression assayed by qRT-PCR with RNA isolated from 3-day-old cultures of PH-1 and pde1 or pde2 mutant. Its relative expression level in PH-1 was arbitrarily set to 1. nd, not detectable.
C. The relative expression level of PDE1 and PDE2 was assayed with RNA isolated from 3-day-old YEPD (arbitrarily set to 1) and LTB (DON-producing) cultures.
D. Three-day-old LTB cultures of the wild type PH-1 and the pde1 and pde2 mutants were examined for bulbous structures (labelled with arrows). Bar = 10 μm.

Fig. 6. Assays for PKA activities and MAP kinase phosphorylation in PDE deletion mutants.
A. PKA activities were assayed with proteins isolated from hyphae of the wild type PH-1, pde1 mutant, pde2 mutant and pde1 pde2 double mutant using the PepTag A1 PKA substrate peptide. Whereas phosphorylated peptides migrated towards the anode (+), un-phosphorylated peptides migrated towards the cathode (-) on a 0.8% agarose gel. N, non-phosphorylated sample control; P, phosphorylated sample control.
B. Western blots of total proteins isolated from vegetative hyphae of the same set of strains were detected with an anti-TpEY antibody for the phosphorylation of Mgv1 (46-kDa) and Gpmk1 (42-kDa) bands. The expression level of Pmk1 was detected with the anti-Pmk1 antibody.

(Supporting Information Fig. S4), indicating that exogenous cAMP may be inhibitory to fungal growth when the cAMP degradation system is abolished.

Although the pde1 and pde2 mutants were normal in perithecia development and ascospore release, the pde1 pde2 double mutant was sterile and failed to develop perithecia 2 weeks post-fertilization (Fig. 4B) or after longer incubation. The double mutant also was significantly reduced in conidiation and virulence (Table 2). It rarely produced conidia and conidia of the double mutant tended to have abnormal morphology (Supporting Information Fig. S5). In infection assays with wheat heads, the pde1 pde2 double mutant has a disease index of less than 1 (Table 2) and caused FHB symptoms only on the inoculated kernels (Fig. 4C), indicating that it was defective in spreading from the inoculation site to nearby spikelets. Therefore, PDE1 and PDE2 must have overlapping functions in hyphal growth, conidiation, sexual development and plant infection in F. graminearum.

We also assayed DON production with the pde1 pde2 double mutant. In comparison with the pde2 mutant, the pde1 pde2 double mutant was increased in DON production in rice grain cultures and diseased wheat kernels (Table 2). These results suggest that, although Pde2 is the
major regulator, Pde1 also plays a minor role in negatively regulating DON production in *F. graminearum*.

Deletion of PDE2 increases PKA activities but reduces MAP kinase activation

To determine the effect of PDE deletion on the activation of PKA, we assayed PKA activities with proteins isolated from hyphae of YEPD cultures. In comparison with PH-1, the *pde2* mutant had a higher PKA activity. Interestingly, although PKA activity was normal in the *pde1* mutant, the double mutant had a higher PKA activity than the *pde2* mutant, indicating that Pde1 also negatively regulates PKA activities, particularly in the absence of Pde2 (Fig. 6A).

To determine the relationship between cAMP signalling and MAP kinase pathways, we assayed the activation of Gpmk1 and Mgv1 in the wild type and PDE deletion mutants. When detected with an anti-MAPK antibody, the 42-kD band had similar intensities in all the strains assayed. However, when detected with an anti-TpEY antibody, phosphorylation of Gpmk1 was significantly reduced in the *pde2* and *pde1 pde2* double mutants (Fig. 6B). Phosphorylation of the 46-kD Mgv1 band also was reduced in the *pde2* and *pde1 pde2* mutants (Fig. 6B). These results indicate that an elevated level of intracellular cAMP may have a negative impact on the activation of Gpmk1 and Mgv1 MAP kinases, which may be related to the defect of *pde2* mutant in plant infection although it was increased in DON production.

The tri10 pde2 but not tri6 pde2 mutant is partially recovered in DON regulation

To confirm different effects of exogenous cAMP on DON production in the *tri6* and *tri10* mutants, we generate the *tri6 pde2* and *tri10 pde2* double mutants (Table 1). Same to the *tri6* mutant, the *tri6 pde2* double mutant failed to produce DON in arginine-containing LTB cultures. In contrast, the *tri10 pde2* double mutant produced significantly more DON than the *tri10* mutant, although not as much as the wild type in 7-day-old arginine-containing LTB cultures (Fig. 7A).

When assayed for TRI gene expression, TRI5 was significantly down-regulated in the *tri6 pde2* and *tri6* mutant in comparison with PH-1 (Fig. 7B). Therefore, deletion of PDE2 could not recover DON production or TRI gene expression in the *tri6* mutant. In contrast, TRI5 expression was significantly increased in the *tri10 pde2* mutant compared to the *tri10* mutant (Fig. 7B). These results further indicate that over-stimulation of the cAMP-PKA pathway could partially suppress the defects of the *tri10* but not *tri6* mutant.

The *tri6 pde2* mutant has increased bulbous structures

Interestingly, although DON biosynthesis was blocked in the *tri6 pde2* mutant, it produced abundant bulbous structures in 3-day-old LTB cultures (Fig. 7C), which is similar to the *pde2* single mutant. Under the same conditions, the wild type strain rarely had bulbous structures (Fig. 7C). Similar to the *pde2* mutant, the *tri10 pde2* double mutant also was increased in the production of intercalary bulbous structures in comparison with the wild type strain (Fig. 7C). These results indicate that cellular differentiation...
Discussion

As a second messenger, cAMP plays a key role in the regulation of morphogenesis, nutrient sensing, secondary metabolism and virulence in various fungal pathogens (Xu and Hamer, 1996; Freitas et al., 2010; Hu et al., 2014). In this study, we showed that DON production was induced by treatment with exogenous cAMP or IBMX that is stimulatory to intracellular cAMP signalling (Fig. 8). Treatment with 4 mM cAMP significantly increased DON production and TRI gene expression. Although the cAMP-PKA pathway has been implicated in the regulation of secondary metabolism in fungal pathogens (Freitas et al., 2010; Studt et al., 2013), to our knowledge significant stimulation of mycotoxin production by exogenous cAMP has not been reported in plant pathogenic fungi. A report on the effect of exogenous cAMP on secondary metabolism is in Monascus species, in which treatment with 10 mM cAMP represses the production of lovastatin, red pigments and citrinin (Miyake et al., 2006). Results from our pharmacological studies with cAMP and IBMX are consistent with earlier publications on the positive regulatory role of the FAC1, CPK1 and CPK2 genes in F. graminearum (Bormann et al., 2014; Hu et al., 2014). However, the cAMP-PKA pathway is known to negatively regulate the biosynthesis of fusarubins and positively regulate bikaverin biosynthesis in Fusarium fujikuroi (Studt et al., 2013).

Interestingly, we observed that exogenous cAMP stimulated the differentiation of bulbous hyphae or intercalary bulbous structures associated with DON production in F. graminearum. In plant pathogenic fungi, cAMP signalling is known to regulate pathogenesis-related morphogenesis (Borges-Walmsley and Walmsley, 2000; D’Souza and Heitman, 2001). In M. oryzae, the cAMP-PKA pathway is important for surface recognition and initiation of appressorium formation (Xu et al., 1997). Exogenous cAMP induces appressorium formation on hydrophobic surfaces (Lee and Dean, 1993). Disruption of cAMP signalling also results in defects in appressorium formation in Colletotrichum lagenarium and Colletotrichum trifoli (Yang and Dickman, 1999; Yamauchi et al., 2004). In F. graminearum, DON is one of the best characterized pathogenicity factors and DON production during plant infection is affected by various environmental and plant factors (Boutigny et al., 2010; Schreiber et al., 2011; Hou et al., 2015; Jiang et al., 2015). It is likely that the regulation of cellular differentiation associated with DON production by cAMP signalling in F. graminearum may involve similar mechanisms regulating pathogenesis-related morphogenesis in other fungal pathogens in response to the physical or chemical signals of host plants.

Although their functional relationship is not clear, TRI6 and TRI10 are two pathway-specific transcriptional regulators of trichothecene biosynthesis. TRI genes expression was blocked or significantly reduced in both tri6 and tri10 deletion mutants (Seong et al., 2009). Interestingly, we found that treatment with exogenous cAMP had different effects on the tri6 and tri10 mutants in DON production (Fig. 8). In the tri10 mutant, cAMP treatment stimulated TRI5 gene expression and DON production, suggesting that overstimulation of the cAMP-PKA pathway could...
partially suppress its defects. This observation was further confirmed by increased DON production in the tri10 pde2 mutant. Deletion of PDE2 resulted in an increase in the intracellular cAMP level, which likely had similar effects as exogenous cAMP in the tri10 mutant background. These results indicate that the regulatory function of TRI10 in trichothecene biosynthesis could be somehow partially bypassed by elevated PKA activities. Therefore, TRI10 may only play a non-essential or indirect role in the regulation of trichothecene biosynthesis by cAMP signalling in F. graminearum. It is likely that TRI10 is not directly activated by PKA or its normal activation involves PKA and other signalling pathways. In F. graminearum, Tri10 has been shown to interact with AreA, a transcription factor that positively regulates TRI gene expression and DON regulation (Hou et al., 2015). AreA is the key regulator of nitrogen metabolism in filamentous fungi. In F. graminearum, the conserved PKA phosphorylation site S874 is important for AreA function and the areA deletion mutant is significantly reduced in DON production, PKA activities, and ammonium permease (MEP) gene expression (Hou et al., 2015). The interaction between Tri10 and AreA may be affected by exogenous cAMP or deletion of PDE2 (Fig. 8). It is also possible that elevated PKA activities may allow AreA to function in the absence of Tri10 in growth or developmental processes that normally require the Tri10-AreA protein complex.

Unlike the tri10 mutant, exogenous cAMP had no effects on the tri6 mutant for DON biosynthesis. DON production also was not detected in the tri6 pde2 mutant. Exogenous cAMP or deletion of PDE2 failed to induce the expression of TRI5 and TRI12 in the tri6 deletion background. Therefore, it is likely that regulation of DON production by cAMP signalling directly involves TRI6. When analysed with NetPhosK 1.0 (Blom et al., 2004), two conserved PKA phosphorylation sites, S61 and S117, were identified in the predicted Tri6 protein. To determine whether TRI6 indeed functions as a direct downstream target of the cAMP-PKA pathway, it will be important to experimentally characterize the PKA-phosphorylation sites in Tri6 and determine their roles in the regulation of DON biosynthesis in F. graminearum. Interestingly, the expression of TRI6 itself was up-regulated when treated with 4 mM cAMP. Binding of Tri6 to its own promoter has been shown to negatively regulate TRI6 expression (Nasmith et al., 2011). It is possible that exogenous cAMP or deletion of PDE2 results in the hyper-phosphorylation of Tri6, which may release its self-inhibitory binding to the TRI6 promoter.

Hydrolysis of cAMP by PDEs plays an important role in regulating the intracellular concentration of this second messenger. In most fungi, the high-affinity PDE play a major role in regulating the basal cAMP level and PKA activities (Ramanujam and Naqvi, 2010; Harren et al., 2013) although the low-affinity PDE appears to be more important in C. neoformans (Hicks et al., 2005). Whereas mutants deleted of the yeast PDE2 ortholog are defective in sexual reproduction and pathogenesis, deletion of the PDE1 ortholog usually had no obvious phenotypes in plant pathogenic fungi M. oryzae and B. cinerea (Ramanujam and Naqvi, 2010; Harren et al., 2013). In F. graminearum, PDE2 plays a more important role than PDE1 in the regulation of trichothecene biosynthesis (Fig. 8). However, PDE1 and PDE2 have redundant functions in conidiation, sexual reproduction and plant infection. Whereas the pde1 and pde2 deletion mutants had no obvious defects, the pde1 pde2 double mutant was significantly reduced in conidiation and virulence and blocked in sexual reproduction in F. graminearum, suggesting that PDE1 and PDE2 are equally important for cAMP phosphodiesterase activities during sexual/asexual differentiation and plant infection. This observation is different from earlier studies with PDE genes in other fungal pathogens (Ramanujam and Naqvi, 2010; Harren et al., 2013). It will be important to determine the molecular mechanisms responsible stage-specific functional redundancy and differences between Pde1 and Pde2 during conidiation, sexual reproduction, DON production and plant infection in F. graminearum.

Deletion of PDE1 had no obvious effects on hyphal growth but the pde2 mutant deletion mutant formed compact colonies and had a slight reduction in growth rate. Therefore, similar to its role in DON biosynthesis, PDE2 likely is more important and PDE1 in vegetative hyphae. Hyphal growth of the pde2 but not pde1 mutant was hypersensitive to exogenous cAMP. further proving that Pde2 plays a major role in cAMP phosphodiesterase activities during vegetative growth. These results indicate that the functional relationship between Pde1 and Pde2 varies in different growth and developmental stages in F. graminearum. It will be important to determine what factors determine when Pde1 and Pde2 have similar functions during conidiation, sexual reproduction and plant infection or when Pde2 plays a major role in cAMP phosphodiesterase activities during vegetative growth and DON production in F. graminearum. One possibility is that the intracellular cAMP level may differ among various cell types, affecting the requirement of Pde1 and Pde2 cAMP phosphodiesterase.

Like treatment with exogenous cAMP, deletion of PDE2 stimulated the formation of intercalary bulbous structures. In F. graminearum, this type of hyphal bulbous structures developed before TRI gene transcription is tightly related to DON production (Jonkers et al., 2012). Similar to the pde2 mutant, the tri6 pde2 and tri10 pde2 double mutants also produced abundant intercalary hyphal bulbous structures. These results indicated that the formation of hyphal bulbous structures stimulated by cAMP or deletion of PDE2 is independent of TRI6 or TRI10. Although it remains possible that TRI6 or TRI10 have redundant roles
in this cellular differentiation, other transcription factors may be involved in regulating the development of hyphal bulbous structures associated with DON production. One candidate transcription factor is Fgp1, the Wor1 ortholog, that functions as a positive regulator of DON synthesis in *F. graminearum* (Jonkers et al., 2012). The *fgp1* deletion mutant rarely forms hyphal bulbous structures associated with DON production. In *C. albicans*, Wor1 is phosphorylated by Tpk2, a subunit of PKA, for activation (Huang et al., 2010). The Fgp1 protein has a conserved PKA phosphorylation site and it may be phosphorylated by PKA in *F. graminearum* as a downstream transcription factor (Fig. 8).

**Experimental procedures**

**Strains and phenotype observation**

All the wild-type and mutant *F. graminearum* strains and transformants generated in this study were listed in Table 1. Potato dextrose agar (PDA) cultures grown at 25°C were used for assaying growth rate and colony morphology. Conidiation was assayed with 5-day-old carboxymethylcellulose (CMC) cultures as described (Hou et al., 2002). For mating assays, aerial hyphae of 7-day-old carrot agar cultures were pressed down with 0.1% Tween 20 and further incubated at 25°C under black light as described (Bowden and Leslie, 1999). Perithecia formation, production of cirrhi, and ascospore release were examined 10–14 days post-fertilization (dpf). CM medium with 2 mM CAMP was used to assay for sensitivities of the *pde* mutants to exogenous CAMP.

**Generation of the *pde1*, *pde2* and *pde1 pde2* deletion mutants**

To generate the gene replacement construct for *PDE1* with the split-marker approach (Catlett et al., 2003), the 0.9-kb upstream and 0.9-kb downstream flanking sequences were amplified by polymerase chain reaction (PCR), connected with the hygromycin phosphotransferase (*hph*) cassette by overlapping PCR, and transformed into protoplasts of PH-1 as described (Hou et al., 2015; Jiang et al., 2015). Hygromycin-resistant transformants were screened by PCR and putative *pde1* deletion mutants were confirmed by Southern blot analysis.

The same approach was used to generate the *PDE2* deletion mutants. To generate the *pde1 pde2* double mutants, the *PDE2* gene replacement construct was generated with the neomycin resistance gene (Jiang et al., 2015) and transformed into the *pde1* mutant. Transformants resistant to both hygromycin and G418 were confirmed by PCR.

For complementation assays, the *PDE2-GFP* fusion construct was generated by the gap repair approach by cotransformation of the *PDE1* gene fragment and Xhol-digested pXY201 (Zhou et al., 2011) into yeast strain XK1-25 as described (Bruno et al., 2004). The resulting *PDE2-GFP* fusion construct recovered from Trp+ yeast transformants was confirmed by sequencing analysis. The same approach was used to generate the *PDE2-GFP* fusion construct with the *pDL2* vector (Zhou et al., 2011). The *PDE1-RFP* and *PDE2-GFP* constructs were then transformed into protoplasts of the *pde1* and *pde2* mutants respectively. Transformants resistant to both hygromycin and bleomycin were screened by PCR and examined for GFP and RFP signals as described (Zhou et al., 2012).

**Plant infection**

Conidia were harvested from 5-day-old CMC cultures and resuspended to a final concentration of 10⁵ spores per milliliter in sterile distilled water as described (Zhou et al., 2010; Jiang et al., 2015). Flowering wheat heads of cultivar Norm were drop-inoculated at the fifth spikelet from the bottom with 10 μl of conidium suspensions as described (Gale et al., 2002). To keep the moisture, inoculated wheat heads were capped with a plastic bag for 48 h. After removing plastic bags, inoculated wheat plants were grown under normal conditions in a greenhouse. Scab symptoms were examined 14 days post-inoculation (dpi) and infected wheat kernels were harvested and assayed for DON production as described (Bluhm et al., 2007).

**Assays for stimulatory effects of cAMP on DON production and TRI gene expression**

For assaying its stimulatory effects on DON production, cAMP (Sigma, USA), were added to a final concentration of 1, 2 and 4 mM to LTB culture as described (Gardiner et al., 2009). IBMX (Sigma, USA) and caffeine (Sigma, USA) were added.
to LTB to the final concentration of 5 mM and 10 mg/ml respectively. DON production in LTB cultures was assayed with a competitive ELISA based DON detection plate kit (Beacon Analytical Systems, Inc, USA) after incubation at 25°C for 1, 3 or 5 days as described (Gardiner et al., 2009).

For assaying TRI gene expression, hyphae were harvested from 3-day-old LTB cultures and used for RNA isolation with the TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized by iScript™ cDNA Synthesis Kit (BIO-RAD, USA) and used for qPCR with an iQ™ Universal SYBR® Green Supermix (BIO-RAD, USA) following manufacturer’s instructions. The beta-tubulin gene of F. graminearum was used as the internal control. Relative expression levels of each gene were calculated with the 2^{-\Delta\Delta Ct} method (Livak and Schmittgen, 2001). The mean and standard deviation were calculated with data from three independent biological replicates.

Assays for TEY phosphorylation and PKA activities

Hyphae were harvested from 48 h YEPD (Yeast extract peptone dextrose) cultures (Zheng et al., 2012) by filtration through two layers of Miracloth (Sigma, USA) and washed with sterile distilled water. Proteins were isolated from vegetative hyphae as described (Zheng et al., 2012). PKA activities were assayed with the PepTag nonradioactive PKA assay kit (Promega, Madison, WI) as described (Adachi and Hamer, 1998). For western blot analyses, total proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Zhou et al., 2012). Phosphorylation of Gpmk1 and Mgv1 was detected with the PhosphoPlus p44/42 MAP kinase antibody kit (Cell Signaling Technology, USA) (Bruno et al., 2004). Detection with an anti-Pmk1 antibody (Bruno et al., 2004) was used as the expression control.

Acknowledgements

We thank Dr. Shijie Zhang, Mr. Qiang Zhang, and Mr. Tao Yin for assistance with DON measurement. We also thank Dr. Jianhua Wang and Dr. Qinhu Wang for bioinformatics analysis. This work was supported by the National Major Project of Breeding for New Transgenic Organisms (2012ZX08009003), Program for New Century Excellent Talents in University (NCET-12-0472), National Natural Science Foundation of China (No. 31271989 and 31301607), Program for New Century Excellent Talents in University (NCET -12-0472), National Natural Science Foundation for Agro-scientific Research in the Public Interest (2012ZX08009003), Program for New Century Excellent Talents in University (NCET-12-0472), National Natural Science Foundation of China (No. 31271989 and 31301607), The Special Fund for Agro-scientific Research in the Public Interest (201303016) and Youth Science & Technology star of Shaanxi (2014KJXX-41). The authors have no conflict of interest to declare.

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Supporting information

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

Fig. S1. Effect of exogenous cAMP on DON production in the *cpk1 cpk2* mutant. DON production was assayed with arginine-containing LTB cultures of the *cpk1 cpk2* double mutant in the presence of 0 or 4 mM cAMP. The wild-type strain PH-1 was used as the control. nd, not detectable.

Fig. S2. Identification of two phosphodiesterase genes in *F. graminearum*.

A. Sequence alignment of the predicted active sites of the low-affinity cAMP phosphodiesterase from *Neurospora crassa*, *B. cinerea*, *Aspergillus nidulans*, *M. oryzae*, *Verticillium dahlia*, *C. albicans*, *S. cerevisiae*, and *F. graminearum*. The lower panel shows the class II PDE consensus sequence.

B. Sequence alignment of the predicted active sites of the high-affinity cAMP phosphodiesterase from marked fungi. The lower panel shows the class I PDE consensus sequence.

Fig. S3. Colony growth and DON production of the *pde2*/PDE2-GFP transformant.

A. Cultures of PH-1 and *pde2*/PDE2-GFP transformant grown on CM for 4 days.

B. DON production in 7-day-old LTB cultures of PH-1 and *pde2*/PDE2-GFP transformant.

Fig. S4. Increased sensitivity of the *pde2* and *pde1 pde2* mutants to exogenous cAMP. Cultures of PH-1, *pde1* mutant, *pde2* mutant and *pde1 pde2* double mutant grown on CM medium with 2 mM cAMP. Photos were taken after incubation for 4 days.

Fig. S5. Conidia of PH-1 and the *pde1, pde2* and *pde1 pde2* mutants. Conidium morphology was examined with conidia harvested from 5-day-old CMC cultures. Bar = 10 μm.