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The AreA transcription factor mediates the regulation of deoxynivalenol (DON) synthesis by ammonium and cyclic adenosine monophosphate (cAMP) signalling in *Fusarium graminearum*

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SUMMARY

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium graminearum*, is harmful to humans and animals. Because different nitrogen sources are known to have opposite effects on DON production, in this study, we characterized the regulatory mechanisms of the *AREA* transcription factor in trichothecene biosynthesis. The Δ*areA* mutant showed significantly reduced vegetative growth and DON production in cultures inoculated with hyphae. Suppression of *TRI* gene expression and DON production by ammonium were diminished in the Δ*areA* mutant. The deletion of *AREA* also affected the stimulatory effects of arginine on DON biosynthesis. The AreA-green fluorescent protein (GFP) fusion complemented the Δ*areA* mutant, and its localization to the nucleus was enhanced under nitrogen starvation conditions. Site-directed mutagenesis showed that the conserved predicted protein kinase A (PKA) phosphorylation site S874 was important for AreA function, indicating that AreA may be a downstream target of the cyclic adenosine monophosphate (cAMP)-PKA pathway, which is known to regulate DON production. We also showed that AreA interacted with Tri10 in co-immunoprecipitation assays. The interaction of AreA with Tri10 is probably related to its role in the regulation of *TRI* gene expression. Interestingly, the Δ*areA* mutant showed significantly reduced PKA activity and expression of all three predicted ammonium permease (*MEP*) genes, in particular *MEP1*, under low ammonium conditions. Taken together, our results show that *AREA* is involved in the regulation of DON production by ammonium suppression and the cAMP-PKA pathway. The AreA transcription factor may interact with Tri10 and control the expression and up-regulation of *MEP* genes.

Keywords: ammonium suppression, DON production, *Gibberella zeae*, nitrogen metabolism, *TRI6* expression.

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INTRODUCTION

Fusarium head blight (FHB) or scab is one of the most important diseases of wheat and barley. *Fusarium graminearum* (teleomorph *Gibberella zeae*) is a major causal agent of FHB in North America and other parts of the world (Bai and Shaner, 2004; Goswami and Kistler, 2004). In addition to causing severe yield losses, this pathogen produces mycotoxins, such as deoxynivalenol (DON) and zearalenone, which are harmful to humans and animals (Desjardins *et al*., 2000). As a trichothecene, DON is a potent inhibitor of protein synthesis in eukaryotic organisms and is also toxic to plant cells (Maier *et al*., 2006). Indeed, DON production was the first and best studied virulence factor in *F. graminearum* (Desjardins *et al*., 2000; Proctor *et al*., 1997).

In the past decade, the genes involved in trichothecene biosynthesis have been well characterized (Desjardins *et al*., 2000). In *F. graminearum*, most of the *TRI* genes are in the core *TRI* cluster, including the trichodiene synthase gene *TRI5* and two transcription factor genes *TRI6* and *TRI10*. *TRI101* and *TRI1* are located on separate chromosomal regions (Alexander *et al*., 2009; Brown *et al*., 2004; Gale *et al*., 2005; Kimura *et al*., 2003). Tri5 catabolizes the cyclization of farnesyl pyrophosphate (FPP), the first step of the trichothecene biosynthesis pathway. Tri6 is a C_2H_2 zinc finger protein that plays a more critical role than Tri10 in DON biosynthesis and plant infection, but both are important for the regulation of *TRI* gene expression (Nasmith *et al*., 2011; Seong *et al*., 2009). Tri6 and Tri10 also regulate the expression of the genes involved in the isoprenoid pathway, which is responsible for the biosynthesis of FPP, a precursor for ergosterol and trichothecene biosynthesis (Seong *et al*., 2009).

In the past decade, various environmental and physiological conditions have been shown to affect DON synthesis in *F. graminearum*, including different nitrogen sources, pH, reactive oxygen species (ROS) and fungicide treatments (Audenaert *et al*., 2010; Gardiner *et al*., 2009a, 2009b; Merhej *et al*., 2011; Montibus *et al*., 2013; Ochiai *et al*., 2007). Similar to its inhibition of secondary metabolism in other fungi, ammonium, a preferred nitrogen source for most fungi, suppresses DON production in

F. graminearum (Pestka *et al*., 1985). However, several nitrogen sources, such as arginine, are known to induce DON biosynthesis (Gardiner *et al*., 2009a). Similar to Gln3 or Gat1 in *Saccharomyces cerevisiae* (Coffman *et al*., 1996; Minehart and Magasanik, 1991), *NIT-2* and *areA* serve as the global regulators of nitrogen metabolism in *Neurospora crassa* and *Aspergillus nidulans*, respectively (Caddick *et al*., 1986; Fu and Marzluf, 1990). AreA orthologues are also known to function as positive regulators of the genes required for the utilization of secondary nitrogen sources in several other filamentous fungi (Christensen *et al*., 1998; Divon *et al*., 2006; Kim and Woloshuk, 2008; Mihlan *et al*., 2003). In *F. graminearum*, deletion of *AREA*, the orthologue of *areA*, resulted in defects in plant infection and trichothecene biosynthesis, but zearalenone production was not affected (Giese *et al*., 2013; Min *et al*., 2012).

Because of the importance of nitrogen metabolism in inducing DON production, in this study, we further characterized the regulatory role of the AreA transcription factor. Deletion of *AREA* led to a decrease in the suppression of *TRI* gene expression and in DON production by ammonium. The putative nuclear localization signal (NLS) sequence NLS3 and a conserved protein kinase A (PKA) phosphorylation site S874 were found to be important for AreA function. We found that the Δ*areA* mutant showed significantly reduced PKA activities, Gpmk1 phosphorylation and expression of all three predicted ammonium permease (*MEP*) genes in *F. graminearum*. Furthermore, we showed that AreA interacted with Tri10 in co-immunoprecipitation assays. The interaction of AreA with Tri10 may be important for its regulation of DON biosynthesis by ammonium sensing in *F. graminearum*.

RESULTS

Ammonium inhibits *AREA* expression and DON production

In filamentous fungi, ammonium is inhibitory to the use of secondary nitrogen sources, which is regulated by AreA and its orthologues (Bolton and Thomma, 2008). To determine the effect of ammonium on *AREA* (FGSG_08634) expression, we isolated RNA from vegetative hyphae of the wild-type strain PH-1 (Table 1), which were harvested from 2-day-old complete medium (CM) cultures and further incubated in medium with 50 mM nitrate or ammonium as the nitrogen source for 1 h. When assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), the expression level of *AREA* in ammonium samples was 20-fold lower than that in nitrate samples (Fig. 1A), suggesting that *AREA* expression was up-regulated in the medium with 50 mM nitrate as the sole nitrogen source compared with the medium with 50 mM ammonium.

Because ammonium is known to inhibit secondary metabolism in fungi (Pestka *et al*., 1985), we added 50 mM ammonium to 21-day-old rice grain cultures of PH-1. DON production was assayed after incubation for another 5 days. In comparison with the control (no ammonium added), ammonium treatment reduced DON production 2.6-fold (Table 2). When assayed in RNA samples isolated from vegetative hyphae of PH-1 cultured in medium with 1 mM nitrate (nitrogen starvation, NS) or 50 mM ammonium (ammonium suppression, AS) as the sole nitrogen source for 6 h, the expression levels of *TRI5* and *TRI6* were 11.4- and 9.4-fold higher, respectively, under NS than AS conditions (Fig. 1B),

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

Name	Brief description	Reference
PH-1	Wild-type	Cuomo *et al*. (2007)
RH12	Δ*areA* deletion mutant of PH-1	This study
AC6	Δ*areA*/*AREA*-GFP transformant of mutant RH12	This study
RL10	Δ*areA*/*AREA*$^{\Delta NLS1}$-GFP transformant of mutant RH12	This study
RL11	Δ*areA*/*AREA*$^{\Delta NLS1}$-GFP transformant of mutant RH12	This study
RL12	Δ*areA*/*AREA*$^{\Delta NLS2}$-GFP transformant of mutant RH12	This study
RL13	Δ*areA*/*AREA*$^{\Delta NLS2}$-GFP transformant of mutant RH12	This study
RE8	Δ*areA*/*AREA*$^{\Delta NLS3}$-GFP transformant of mutant RH12	This study
RE15	Δ*areA*/*AREA*$^{\Delta NLS3}$-GFP transformant of mutant RH12	This study
RP2	Δ*areA*/*AREA*$^{\Delta S874}$-GFP (PKA site) transformant of RH12	This study
RP4	Δ*areA*/*AREA*$^{\Delta S874}$-GFP (PKA site) transformant of RH12	This study
RM3	Δ*areA*/*AREA*$^{\Delta S657\text{-}S658}$-GFP (MAPK site) transformant of RH12	This study
RM6	Δ*areA*/*AREA*$^{\Delta S657\text{-}S658}$-GFP (MAPK site) transformant of RH12	This study
RPA6	Δ*areA*/*AREA*^{S874A}-GFP (PKA site) transformant of RH12	This study
RPA8	Δ*areA*/*AREA*^{S874A}-GFP (PKA site) transformant of RH12	This study
AT61	P_{RP27}-*AREA*-3 × FLAG and *TRI6*-GFP transformant of PH-1	This study
AT63	P_{RP27}-*AREA*-3 × FLAG and *TRI6*-GFP transformant of PH-1	This study
AT02	P_{RP27}-*AREA*-3 × FLAG and *TRI10*-GFP transformant of PH-1	This study
PH1Δ*tri6*	Δ*tri6* deletion mutant of PH-1	Seong *et al*. (2009)
PH1Δ*tri10*	Δ*tri10* deletion mutant of PH-1	Seong *et al*. (2009)
6N5	Δ*tri6*/*TRI6* complementation transformant of mutant RH12	This study

Table 2 Growth rate, conidiation and deoxynivalenol (DON) production in the Δ*areA* mutant.

Strain	Growth rate (mm/day)†	Conidiation (×10^5/mL)‡	DON/Erg§	
			Control	50 mM NH_4^+
PH-1 (WT)	6.9 ± 0.1^A*	10.5 ± 1.1^A	0.13 ± 0.03^A	0.05 ± 0.01^A
RH12 (Δ*areA*)	5.8 ± 0.0^B	1.9 ± 0.2^B	0.02 ± 0.01^B	0.25 ± 0.09^B
AC6 (Δ*areA*/*AREA*)	7.0 ± 0.1^A	10.2 ± 1.8^A	0.11 ± 0.05^A	0.06 ± 0.02^A

WT, wild-type.

*Data from three replicates were analysed with the protected Fisher's least-significant difference (LSD) test. The same letter indicates that there is no significant difference ($P = 0.05$).

†Growth rate was assayed with 3-day-old complete medium (CM) agar cultures.

‡Conidiation was measured with 5-day-old CarboxyMethylCellulose (CMC) cultures.

§DON/ergosterol (Erg) ratio was assayed with 3-week-old rice grain cultures with or without 50 mM ammonium phosphate added at day 5. Erg was measured to quantify fungal biomass.

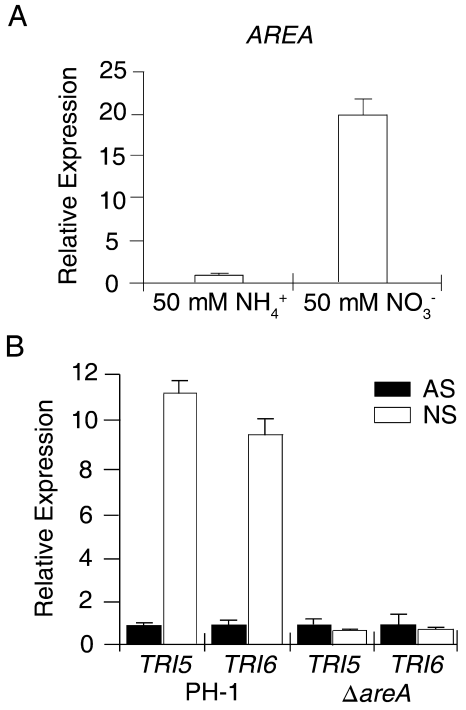

Fig. 1 *AREA* expression and its role in the regulation of the *TRI* gene. (A) Relative expression level of *AREA* in complete medium (CM) cultures with 50 mM ammonium (NH_4^+; arbitrarily set to unity) or nitrate (NO_3^-) as the sole nitrogen source. (B) The expression level of *TRI5* and *TRI6* in cultures of the wild-type strain PH-1 and Δ*areA* mutant grown under nitrogen starvation (NS) or ammonium suppression (AS; arbitrarily set to unity) conditions.

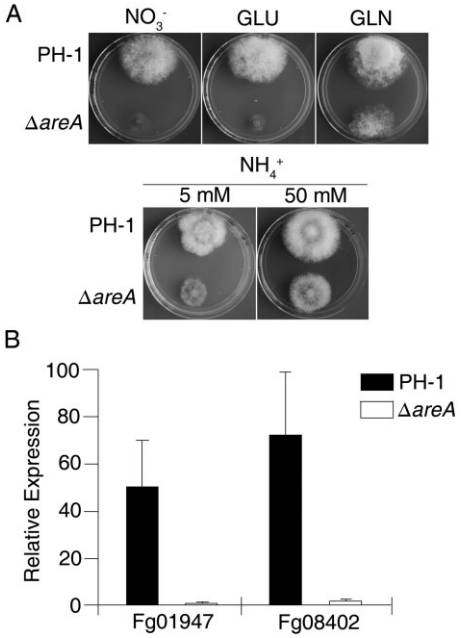

Fig. 2 Defects of the Δ*areA* mutant in response to different nitrogen sources. (A) Cultures of the wild-type strain PH-1 and Δ*areA* mutant grown on complete medium (CM) with 50 mM sodium nitrate (NO_3^-), glutamate (GLU) or glutamine (GLN) and 5 or 50 mM ammonium phosphate (NH_4^+) as the nitrogen source. (B) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays of the nitrate (FGSG_01947) and nitrite (FGSG_08402) reductase genes in PH-1 and Δ*areA* mutant grown under nitrogen starvation (NS) and ammonium suppression (AS; arbitrarily set to 1) conditions.

confirming the inhibitory role of ammonium on trichothecene synthesis.

Inhibitory effect of NH_4^+ on DON production and utilization of secondary nitrogen sources requires *AREA*

We generated the *AREA* gene replacement mutant of the wild-type strain PH-1 (Table 1) by the split-marker approach. Similar to the Δ*areA* mutant of strain GZ03639 (Min *et al*., 2012), the Δ*areA* mutant of PH-1 showed a significantly reduced growth rate (Fig. S1, see Supporting Information), virulence (Fig. S2, see Supporting Information), conidiation and DON production (Table 2). In the absence of other nitrogen sources, high concentrations of ammonium and glutamine, but not nitrate, partially recovered the growth defects of the Δ*areA* mutant (Fig. 2A; Table 3). Furthermore, we found that the growth rate of Δ*areA* increased with an

Table 3 Growth rates of PH-1 and Δ*areA* mutant in cultures with different nitrogen sources and different concentrations of ammonium.

	Growth rate (mm/day)†					
	NH_4^+					
Strain	1 mM	10 mM	100 mM	NO_3^-	Glutamine	Glutamate
PH-1 (WT)	$3.5 \pm 0.1^{A*}$	3.5 ± 0.1^{A}	4.1 ± 0.1^{A}	3.9 ± 0.1^{A}	4.4 ± 0.2^{A}	3.8 ± 0.1^{A}
RH12 (Δ*areA*)	2.0 ± 0.0^{B}	2.0 ± 0.1^{B}	3.4 ± 0.2^{B}	1.8 ± 0.1^{B}	2.7 ± 0.1^{B}	1.3 ± 0.1^{B}

WT, wild-type.

*Data from three replicates were analysed with the protected Fisher's least-significant difference (LSD) test. Different letters are used to mark statistically significant differences ($P = 0.05$).

†Growth rates on modified complete medium (CM) with different nitrogen sources or different concentrations of ammonium were measured after incubation for 3 days.

increase in concentration of ammonium from 5 to 50 mM (Fig. 2A; Table 3).

In rice grain cultures, the addition of ammonium reduced DON production in the wild-type (Table 2), which is consistent with the inhibitory effect of ammonium on secondary metabolism (Teichert *et al*., 2008). However, the Δ*areA* mutant showed more than an 11.4-fold increase in DON biosynthesis in cultures supplemented with 50 mM ammonium compared with regular rice grain cultures (Table 2). These results suggest that, in addition to the stimulatory effect of ammonium on growth, deletion of *AREA* may release its inhibitory effect on DON biosynthesis. To test this hypothesis, we assayed the expression levels of *TRI5* and *TRI6* by qRT-PCR. In the Δ*areA* mutant, *TRI5* and *TRI6* expression was not significantly changed (less than 1.4-fold) in AS cultures relative to NS cultures (Fig. 1B). Therefore, *AREA* is important for the suppression of *TRI5* and *TRI6* expression by ammonium.

Because AreA orthologues are important for the utilization of secondary nitrogen sources, we also assayed the expression of the nitrate reductase (FGSG_01947) and nitrite reductase (FGSG_08402) genes in vegetative hyphae of PH-1 and the Δ*areA* mutant incubated under NS or AS conditions for 6 h. In the wild-type strain, the expression levels of FGSG_01947 and FGSG_08402 increased 49.4- and 74.6-fold, respectively, in NS cultures relative to AS cultures (Fig. 2B). However, NS failed to induce the expression of the nitrate and nitrite reductase genes in the Δ*areA* mutant (Fig. 2B).

Induction of DON biosynthesis by arginine is affected by *AREA* deletion

Because arginine can stimulate DON production, we assayed *TRI* gene expression in liquid cultures with 5 mM nitrate or arginine as described by Gardiner *et al*. (2009a). In PH-1, the expression levels of *TRI5*, *TRI6* and *TRI10* increased 79.3-, 205.9- and 42.3-fold, respectively, in arginine cultures relative to nitrate cultures (Fig. 3A). In the Δ*areA* mutant RH12, the expression levels of *TRI5*, *TRI6* and *TRI10* were induced by arginine to 5.1-, 12.7- and

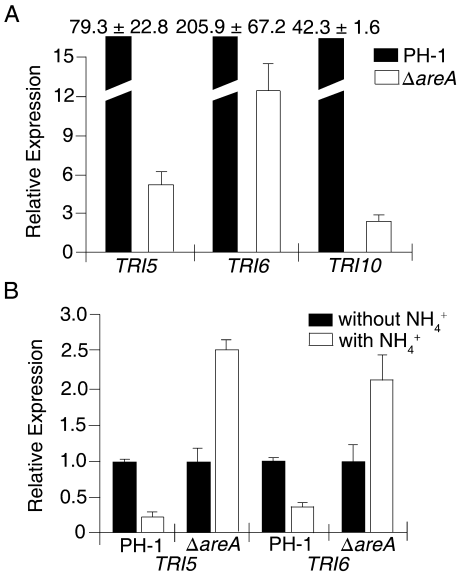

Fig. 3 Stimulation of deoxynivalenol (DON) production by arginine is affected by *AREA*. (A) The expression levels of *TRI5*, *TRI6* and *TRI10* in the wild-type strain PH-1 and Δ*areA* mutant in liquid DON-inducing medium with 5 mM nitrate or arginine. For each gene, the expression level in nitrate cultures was arbitrarily set to unity. (B) The expression levels of *TRI5* and *TRI6* in DON-inducing liquid cultures (with arginine) of PH-1 and Δ*areA* mutant with 0 or 50 mM ammonium added at the fourth day. Mean and standard deviations were calculated with results from three independent biological replicates.

2.6-fold, respectively (Fig. 3A). DON production in arginine cultures of PH-1 and mutant RH12 was also 379.3- and 96.1-fold higher, respectively, than in nitrate cultures (Table 4). These results indicate that the Δ*areA* mutant is still induced by arginine for *TRI* gene expression and DON production, although to a much lesser degree in comparison with the wild-type. Therefore, *AREA* is important, but not essential, for arginine-induced DON biosynthesis.

To test whether it can suppress the stimulatory effect of arginine on DON biosynthesis, ammonium was added to 4-day-old DON-inducing cultures to a final concentration of 50 mM. After

Table 4 Deoxynivalenol (DON) production in liquid cultures of the wild-type (WT) and Δ*areA* mutant.

	DON production (ppm)†	
Strain	$NaNO_3$	Arginine
PH-1 (WT)	$6.2 \pm 2.1^{A*}$	2348.2 ± 156.8^{A}
RH12 (Δ*areA*)	1.7 ± 0.4^{B}	164.6 ± 32.5^{B}

*Data from three replicates were analysed with the protected Fisher's least-significant difference (LSD) test. Different letters are used to mark statistically significant differences ($P = 0.05$).
†DON production in liquid medium with 5 mM $NaNO_3$ or arginine as the nitrogen source was assayed as described by Gardiner *et al*. (2009a).

incubation for another 3 days, the expression level of *TRI5* and *TRI6* in the wild-type strain PH-1 decreased 4.0- and 2.7-fold, respectively, in ammonium-treated samples compared with the controls (Fig. 3B). However, ammonium failed to inhibit the expression of these *TRI* genes in the Δ*areA* mutant. In contrast, *TRI5* and *TRI6* expression was increased 2.6- and 2.1-fold in ammonium-treated samples (Fig. 3B), indicating that *AREA* is important for the inhibitory effects of ammonium on DON biosynthesis induced by arginine.

Localization of AreA-green fluorescent protein (GFP) fusion to the nucleus is enhanced under NS conditions

For complementation assays, *AREA*-GFP fusion constructs were generated and introduced into the Δ*areA* deletion mutant RH12. The resulting Δ*areA*/*AREA*-GFP transformant AC6 (Table 1) was rescued in all the defects of Δ*areA*, including growth and plant infection (Figs S1 and S2). In transformant AC6, weak GFP signals were observed in the nucleus in conidia, germ tubes and vegetative hyphae. To determine the effects of nitrogen sources on AreA-GFP, conidia (Fig. 4A) and hyphae (Fig. 4B) of the Δ*areA*/*AREA*-GFP transformant were cultured under NS conditions for 1 h. In both conidia and hyphae, GFP signals in the nucleus were stronger under NS treatment conditions (Fig. 4), indicating that NS increased the expression and nuclear localization of AreA-GFP fusion proteins.

Functional characterization of putative NLS sequences of AreA proteins

The PIKSRKE (NLS1), RKRP (NLS2) and LHGVVRPLSL (NLS3) sequences of AreA are three putative NLS sequences (Fig. 5A) that are conserved in its orthologues from *Fusarium verticillioides*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *N. crassa* and *A. nidulans* (Hunter *et al*., 2014). To determine their functions in the localization of AreA to the nucleus, we generated the

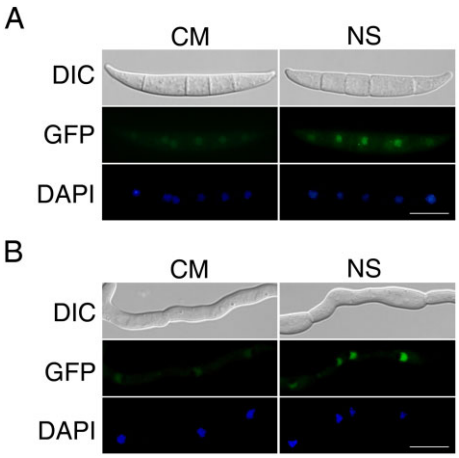

Fig. 4 Expression and subcellular localization of AreA-GFP fusion proteins. (A) Conidia of the Δ*areA*/*AREA*-GFP transformant AC6 were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by differential interference contrast (DIC) or epifluorescence microscopy with or without incubation for 1 h under nitrogen starvation (NS) conditions. (B) Germlings of transformant AC6 were stained with DAPI and examined by DIC or epifluorescence microscopy with or without incubation for 1 h under NS conditions. CM, complete medium; GFP, green fluorescent protein. Bar, 10 μm.

AREA$^{\Delta NLS1}$-GFP, *AREA*$^{\Delta NLS2}$-GFP and *AREA*$^{\Delta NLS3}$-GFP constructs and transformed them into the Δ*areA* mutant RH12. The resulting Δ*areA*/*AREA*$^{\Delta NLS1}$-GFP and Δ*areA*/*AREA*$^{\Delta NLS2}$-GFP transformants (Table 1) showed the wild-type growth rate and produced normal colonies (Fig. 5B). GFP signals were mainly observed in the nucleus in these transformants under different growth conditions, indicating that deletion of NLS1 or NLS2 had no effect on the function and nuclear localization of AreA proteins (Fig. S3, see Supporting Information).

However, the Δ*areA*/*AREA*$^{\Delta NLS3}$-GFP transformants RE8 and RE15 showed partially recovered growth rates (Fig. 5B), although plant infection was still defective (Fig. 5C). When examined under an epifluorescence microscope, we failed to observe GFP signals in the nucleus in conidia or hyphae of these transformants, even under NS conditions (Fig. 5D). On Western blots of proteins isolated from transformants RE8 and RE15, a 130-kDa band was detected with an anti-GFP antibody (Fig. 5E). When assayed as described by Gardiner *et al*. (2009a), the *AREA*$^{\Delta NLS1}$ and *AREA*$^{\Delta NLS2}$ transformants were normal, but the *AREA*$^{\Delta NLS3}$ transformants RE8 and RE15 were only partially recovered in DON production in liquid cultures (Table S2, see Supporting Information). These results indicate that AreA$^{\Delta NLS3}$-GFP fusion proteins were expressed, but not localized, to the nucleus. Therefore, this bipartite NLS sequence must be essential for the localization of AreA to the nucleus and its function during plant infection and DON production in *F. graminearum*.

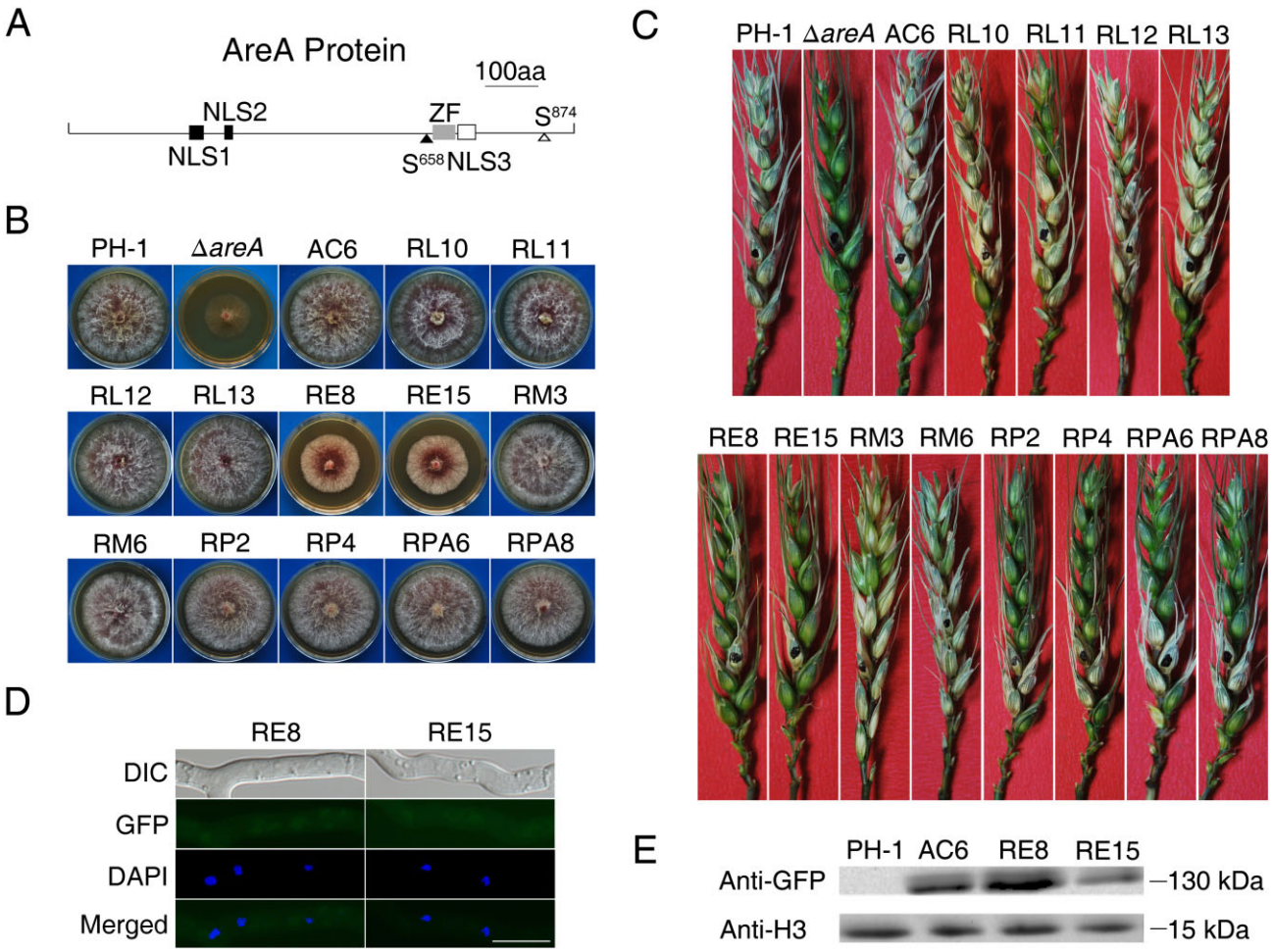

Fig. 5 Site-directed mutagenesis analysis with the *AREA* gene. (A) The AreA protein is predicted to have three conserved nuclear localization signal (NLS) sequences (NLS1, 238–244; NLS2, 287–290; NLS3, 716–725), one consensus protein kinase A (PKA) (S874) and one mitogen-activated protein kinase (MAPK) (S658) phosphorylation site, and one zinc figure domain (ZF, 686–710). (B) Four-day-old potato dextrose agar (PDA) cultures of the wild-type strain PH-1, Δ*areA* mutant, Δ*areA/AREA*$^{\Delta NLS1}$-GFP (RL10 and RL11), Δ*areA/AREA*$^{\Delta NLS2}$-GFP (RL12 and RL13), Δ*areA/AREA*$^{\Delta NLS3}$-GFP (RE8 and RE15), Δ*areA/AREA*$^{\Delta S874}$-GFP (RP2 and RP4), Δ*areA/AREA*$^{\Delta S657\text{-}658}$-GFP (RM3 and RM6) and Δ*areA/AREA*^{S874A}-GFP (RPA6 and RPA8) transformants, and complementation strain (AC6). (C) Wheat heads inoculated with the same set of strains were examined for scab symptoms at 14 days post-inoculation (dpi). (D) Germlings of transformants RE8 and RE15 were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by differential interference contrast (DIC) or epifluorescence microscopy. GFP, green fluorescent protein. Bar, 10 μm. (E) Western blot analysis with total proteins isolated from vegetative hyphae of PH-1 and Δ*areA/AREA*$^{\Delta NLS3}$-GFP transformants RE8 and RE15. The AreA-GFP band was detected with the anti-GFP antibody. Detection with an anti-H3 antibody was used as the control to show that similar amount of proteins were loaded in each lane.

S874 is a putative PKA phosphorylation site important for *AREA* function

Sequence analysis revealed that the AreA protein has one putative consensus mitogen-activated protein kinase (MAPK) phosphorylation site (PSS^{658}P) and two putative optimal PKA phosphorylation sites (S^{32} and S^{874}) (Fig. 5A). However, only the S^{658} and S^{874} residues are well conserved in *AREA* orthologues from *M. oryzae*, *A. nidulans* and *N. crassa*. To determine the role of these MAPK and PKA phosphorylation sites, we generated the *AREA*$^{\Delta S657\text{-}658}$-GFP and *AREA*$^{\Delta S874}$-GFP constructs and transformed them into the Δ*areA* deletion mutant RH12. The Δ*areA/AREA*$^{\Delta S874}$ (RP2 and RP4) and Δ*areA/AREA*$^{\Delta S657\text{-}658}$ (RM3 and RM6) transformants showed similar growth rates to PH-1 (Fig. 5B). In infection assays with flowering wheat heads, transformants RM3 and RM6 were as virulent as the wild-type and the complementation transformant. However, transformants RP2 and RP4 were more virulent than the Δ*areA* mutant, but not as virulent as the wild-type (Fig. 5C), suggesting that *AREA*$^{\Delta S874}$ only partially complemented the defects of Δ*areA* in plant infection.

To further confirm that S874 is important for AreA function during plant infection, we generated the *AREA*^{S874A}-GFP construct

and transformed it into the Δ*areA* deletion mutant RH12. Similar to the *AREA*$^{\Delta S874}$ transformants, the Δ*areA/AREA*^{S874A}-GFP transformants RPA6 and RPA8 showed fully recovered growth rates (Fig. 5B), but only partially rescued virulence in infection assays with flowering wheat heads (Fig. 5C). Nevertheless, the *AREA*$^{\Delta S874}$ and *AREA*^{S874A} transformants were similar to the wild-type and complementation transformant with regard to DON production (Table S2). In these transformants, GFP signals were also mainly observed in the nucleus, but localization of the GFP fusion proteins was not increased by NS (data not shown). These results indicate that S874 is not important for the expression and subcellular localization of AreA, but is important for its function during plant infection.

Expression of ammonium permease genes is regulated by AreA

AreA orthologues are known to regulate the expression of ammonium permease genes in *A. nidulans* (Monahan *et al*., 2006) and *S. cerevisiae* (Magasanik and Kaiser, 2002). In *F. graminearum*, all three predicted ammonium permease genes, FGSG_02094 (*MEP1*), FGSG_00620 (*MEP2*) and FGSG_00529 (*MEP3*), have multiple GATA sequences as putative AreA-binding sites in their promoter regions (Fig. S4, see Supporting Information). When assayed by qRT-PCR, the expression levels of *MEP1*, *MEP2* and *MEP3* were significantly reduced in the Δ*areA* mutant compared with the wild-type in low-concentration ammonium (0.5 mM) cultures (Fig. 6A). In comparison with PH-1, the expression levels of *MEP1*, *MEP2* and *MEP3* were reduced 2275-, 134- and 904-fold in the Δ*areA* mutant in low-concentration ammonium (0.5 mM) cultures (Fig. 6A). Even in high-concentration ammonium (50 mM) cultures, the expression levels of *MEP1*, *MEP2* and *MEP3* were reduced 2.2-, 3.1- and 23.5-fold, respectively, in the Δ*areA* mutant compared with PH-1 (Fig. 6A).

In the wild-type, the expression levels of *MEP1*, *MEP2* and *MEP3* were 1003.3-, 120.2- and 97.3-fold higher, respectively, in low-concentration ammonium cultures than in high-concentration ammonium cultures, suggesting that *MEP1* was the *MEP* gene with the most significant up-regulation by low-concentration ammonium conditions. In the Δ*areA* mutant, *MEP1* expression was not affected by high or low concentrations of ammonium (Fig. 6B). The expression levels of *MEP2* and *MEP3* were increased 2.8- and 2.5-fold under low-concentration relative to high-concentration ammonium conditions (Fig. 6B). Therefore, deletion of *AREA* also affected the up-regulation of *MEP2* and *MEP3*, although to a much lesser degree than *MEP1*, by low-concentration ammonium conditions.

PKA activities and Gpmk1 phosphorylation are reduced in the Δ*areA* mutant

It has been shown that both the cyclic adenosine monophosphate (cAMP)-PKA and MAPK pathways function downstream from Ras signalling in fungi (Li *et al*., 2012; Zhao and Xu, 2007), and Ras proteins may interact with the C-terminal region of *MEP2* in *S. cerevisiae* (Lorenz and Heitman, 1998) and *Candida albicans* (Biswas and Morschhauser, 2005). It is possible that the MEP–RAS association may activate the PKA or MAPK pathway, which, in turn, activates the downstream AreA transcription factor in *F. graminearum*. To test this hypothesis, we assayed the phosphorylation level of Gpmk1. When detected with an anti-TpEY antibody, the Δ*areA* mutant showed a significantly reduced phosphorylation level of the Gpmk1 MAPK, but no obvious changes in Mgv1 activation (Fig. 7A). We

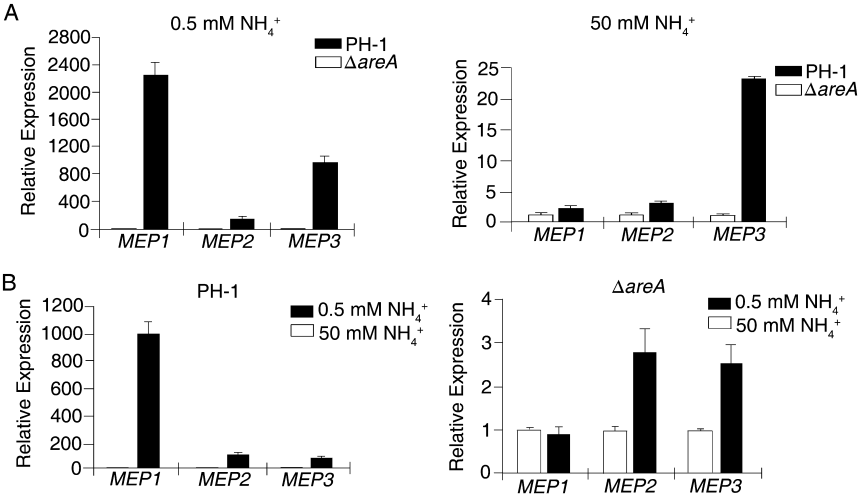

Fig. 6 Assays for the expression levels of three ammonium permease genes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (A) The expression levels of *MEP1*, *MEP2* and *MEP3* were compared between the wild-type strain PH-1 and Δ*areA* mutant, which were cultured in medium with 0.5 mM (low) or 50 mM (high) ammonium. For each gene, the relative expression level in the Δ*areA* mutant was arbitrarily set to unity. (B) The expression level of each *MEP* gene in cultures of PH-1 or Δ*areA* mutant with 0.5 mM ammonium was compared with that of cultures with 50 mM ammonium (arbitrarily set to unity). Mean and standard deviation were calculated with data from three independent biological replicates.

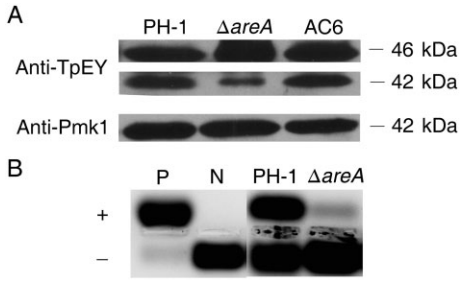

Fig. 7 Mitogen-activated protein kinase (MAPK) phosphorylation and protein kinase A (PKA) activity assays. (A) Assays for the activation of Mgv1 and Gpmk1 MAPKs. Total proteins were isolated from vegetative hyphae of the wild-type PH-1, Δ*areA* mutant and complementation strain (AC6). The phosphorylation of Mgv1 (46 kDa) and Gpmk1 (42 kDa) was detected with the anti-TpEY antibody. The expression level of Gpmk1 was detected with the anti-Pmk1 antibody. (B) PKA activities were assayed with proteins isolated from hyphae of PH-1 and Δ*areA* mutant using the PepTag A1 PKA substrate peptide. Whereas phosphorylated peptides migrated towards the anode (+), unphosphorylated peptides migrated towards the cathode (–) on a 0.8% agarose gel. N, non-phosphorylated sample control; P, phosphorylated sample control.

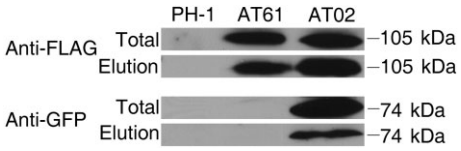

Fig. 8 Co-immunoprecipitation assays for the interactions between Tri10 and AreA. Total proteins (Total) isolated from vegetative hyphae of PH-1, transformant AT61 (expressing the *AREA*-3 × FLAG and *TRI6*-GFP constructs) and transformant AT02 (expressing the *AREA*-3 × FLAG and *TRI10*-GFP constructs) and proteins eluted (Elution) from anti-FLAG M2 beads were separated on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After transfer to nitrocellulose membranes, the presence of AreA-3 × FLAG, Tri10-GFP or Tri6-GFP fusion proteins was detected with the anti-FLAG and anti-GFP antibodies. Whereas the Tri6-GFP band was not detectable in transformant AT61, the 74-kDa Tri10-GFP band was detected in total proteins and proteins eluted from anti-FLAG beads in transformant AT02. GFP, green fluorescent protein.

also assayed PKA activities with proteins isolated from vegetative hyphae of yeast extract peptone dextrose (YEPD) cultures as described by Adachi and Hamer (1998). In comparison with PH-1, the Δ*areA* mutant showed significantly reduced PKA activities (Fig. 7B). These data indicate that deletion of *AREA* may affect the cAMP-PKA pathway and the activation of Gpmk1 MAPK in *F. graminearum*.

AreA interacts with Tri10 *in vivo*

One possibility for the regulation of DON production by AreA is that it may interact with Tri6 or Tri10, two key transcription factors in the *TRI* cluster. To test this hypothesis, we first attempted to study their interactions by yeast two-hybrid assays, but Tri6, Tri10 and AreA all showed strong self-activation activities. Therefore, we generated the P_{RP27}-*TRI6*-GFP and P_{RP27}-*TRI10*-GFP constructs and co-transformed them into PH-1 with P_{RP27}-*AREA*-3 × FLAG. Because GFP signals were not observed in transformants expressing the *TRI6*-GFP and *TRI10*-GFP constructs that complemented the Δ*tri6* and Δ*tri10* deletion mutants (Seong *et al*., 2009), we used the RP27 promoter, a strong, constitutive promoter, to overexpress these two genes. Unfortunately, we detected the AreA-3 × FLAG band, but failed to detect the Tri6-GFP band, in the transformant AT61 (Fig. 8), which was confirmed by PCR analysis to contain the P_{RP27}-*TRI6*-GFP and *AREA*-3 × FLAG fusion constructs. Therefore, it is impossible to assay the interaction between AreA and Tri6.

In total proteins isolated from the P_{RP27}-*TRI10*-GFP *AREA*-3 × FLAG transformant AT02, both the 105-kDa AreA-3 × FLAG band and the 74-kDa Tri10-GFP band were detected with the anti-FLAG and anti-GFP antibodies, respectively (Fig. 8). The same antibodies failed to detect these two bands in proteins isolated from the wild-type strain PH-1 (Fig. 8). In proteins eluted from anti-FLAG beads, both AreA-3 × FLAG and Tri10-GFP fusion proteins were detected in transformant AT02 (Fig. 8) over three independent replicates. Under the same conditions, only the AreA-3 × FLAG band was detected in proteins eluted from anti-FLAG beads for transformant AT61. These data indicate that AreA interacts with Tri10 in *F. graminearum*, which may be important for the function of AreA in the regulation of DON biosynthesis.

DISCUSSION

AreA orthologues are well conserved in filamentous ascomycetes for the regulation of the utilization of secondary nitrogen sources (Tudzynski, 2014). In *F. graminearum*, *AREA* is required for the induced expression of the nitrate and nitrite reductase genes when nitrate is the sole nitrogen source, which is similar to *FNR1* in *F. oxysporum* and *AREA* orthologues in other fungi (Divon *et al*., 2006; Feng and Marzluf, 1998). The Δ*areA* mutant is defective in the utilization of nitrate or glutamate as the sole nitrogen source and shows limited growth, which is similar to the *areA* mutant of *A. oryzae* (Christensen *et al*., 1998).

Because of its importance in secondary metabolism, we had started to functionally characterize the AreA orthologue before the publication on AreA in a different isolate of *F. graminearum* (Min *et al*., 2012). Similar to the earlier report (Min *et al*., 2012), we found that the Δ*areA* mutant was defective in growth and conidiation. Although at a significantly reduced level, the Δ*areA* mutant still produced DON in rice grain cultures or DON-inducing liquid cultures when hyphae were used as the inoculum. It also showed reduced suppression of DON production and reduced expression of *TRI5* and *TRI6* by ammonium. These results indicate that *AREA* is important, but not essential, for DON biosynthesis

and the suppression of DON biosynthesis by ammonium. As a result of its defects in hyphal growth, the Δ*areA* mutant was defective in DON production in DON-inducing liquid medium inoculated with conidia, which is similar to the results reported by Min *et al*. (2012).

Although AreA orthologues are known to be important for AS of secondary metabolism, such as fumonisin biosynthesis in *F. verticillioides* (Kim and Woloshuk, 2008) and gibberellin synthesis in *Gibberella fujikuroi* (Mihlan *et al*., 2003; Teichert *et al*., 2004), the underlying mechanisms are not well characterized. In this study, we showed that AreA interacted with Tri10 in co-immunoprecipitation assays. Tri10 and Tri6 are two transcription factors in the *TRI* gene cluster important for the regulation of *TRI* gene expression and DON biosynthesis (Seong *et al*., 2009). It is possible that the interaction of Tri10 with AreA is important for the induction of *TRI* gene expression. The suppression of *AREA* expression by ammonium may be directly responsible for its inhibitory effects on DON production.

In *G. fujikuroi*, the AreA orthologue appears to mediate gibberellin (GA) production by binding to the promoters of the GA biosynthesis genes (Mihlan *et al*., 2003; Teichert *et al*., 2004; Tudzynski *et al*., 1999). The promoter sequences of *TRI6*, *TRI10* and other *TRI* genes all have multiple 5'-HGATAR-3' sequences that may function as putative AreA-binding sites (Ravagnani *et al*., 1997; Scazzocchio, 2000). It is possible that AreA may directly regulate the expression of some of these *TRI* genes. However, the HGATAR sequence occurs randomly at least once in every 256 bp (both directions). The promoter of *TRI6* alone has six putative AreA-binding sites. Therefore, it is difficult to predict and functionally identify which is the actual AreA-binding site in the *TRI* genes of *F. graminearum*. In the future, it will be important to conduct chromatin immunoprecipitation sequencing (ChIP-seq) analysis to identify promoter sequences of *TRI* genes binding to AreA in *F. graminearum*.

In *F. graminearum*, the expression of *AREA* was repressed by ammonium and up-regulated when 50 mM nitrate was the sole nitrogen source. In the *AREA*-GFP transformant, AreA-GFP constitutively localized to the nucleus, although GFP signals in the nucleus were enhanced when nitrate was the sole nitrogen source, which is consistent with its role in the positive regulation of nitrate assimilation genes. In *A. nidulans*, AreA is located primarily in the cytoplasm when ammonium is present, but is accumulated in the nucleus in response to NS (Todd *et al*., 2005). In *S. cerevisiae*, Gln3 localizes to the nucleus to positively regulate the expression of nitrogen catabolite repression (NCR)-sensitive genes (Beck and Hall, 1999). Therefore, the subcellular localization of AreA-GFP is slightly different from its orthologues in *A. nidulans* and *S. cerevisiae*.

NLS1 and NLS2 are two well-conserved NLS sequences in AreA and its orthologues. However, deletion of either NLS1 or NLS2 of AreA had no obvious effect on its localization and function in *F. graminearum*. In *A. nidulans*, mutations at these two NLS sites also had no effect on the subcellular localization of AreA (Hunter *et al*., 2014). However, AreA has a non-canonical arginine-based bipartite NLS sequence that is important for its nuclear localization. This NLS sequence is conserved in most fungi, including *F. graminearum*. To avoid interfering with the nearby zinc finger DNA-binding domain, only part of this NLS sequence was deleted in the *AREA*$^{\Delta NLS3}$ allele. Our data showed that AreA$^{\Delta NLS3}$-GFP proteins were detected by Western blot analysis, but no GFP signals were observed in the nucleus. Therefore, NLS3 must be the NLS sequence that is responsible for the subcellular localization of AreA in *F. graminearum*. In *A. nidulans*, mutations in the four R residues in this NLS also significantly reduced the localization of AreA to the nucleus (Hunter *et al*., 2014). In *F. graminearum*, no detectable GFP signals were observed in the *AREA*$^{\Delta NLS3}$-GFP transformant, even under NS conditions.

The AreA protein has a putative PKA phosphorylation site that is well conserved among its orthologues from filamentous ascomycetes. Site-directed mutagenesis showed that this PKA phosphorylation site was important for the increased localization of AreA-GFP to the nucleus and full virulence, suggesting the importance of this consensus PKA site. In *S. cerevisiae*, phosphorylation of Gln3 is dependent on nitrogen sources in the medium. In the presence of primary nitrogen sources, Gln3 is phosphorylated by the Tor1 and Tor2 TOR kinases (Beck and Hall, 1999; Bertram *et al*., 2000). The Snf1 and Npr1 kinases are also probably involved in Gln3 regulation (Bertram *et al*., 2002; Cox *et al*., 2002; Crespo *et al*., 2004). In *A. nidulans*, the regulation of AreA phosphorylation is different from that in *S. cerevisiae* (Todd *et al*., 2005), and it is not known to involve PKA. Direct phosphorylation of AreA orthologues by PKA has also not been reported in other fungi. Therefore, further studies are necessary to determine whether S874 is phosphorylated by Cpk1 or Cpk2, although it is a conserved, consensus PKA phosphorylation site based on bioinformatics analysis. Cpk1 and Cpk2 are two catalytic subunits of PKA that are essential for DON production and plant infection in *F. graminearum* (Hu *et al*., 2014).

In *S. cerevisiae*, the Gln3 and Nil1 GATA factors mediate NCR. They control the expression of three ammonium permease genes, named *MEP1*, *MEP2* and *MEP3* (Marini *et al*., 1997). In *A. nidulans*, the expression of all three *AMT/MEP* is also regulated by AreA under different nitrogen conditions. The AreA/Nit-2 global nitrogen regulator binds specifically to GATA sequences in the promoters of its target genes to regulate their expression. In this study, we found that all three predicted *F. graminearum MEP* genes have several GATA sequences in the promoter regions, and their expression was significantly reduced in the Δ*areA* mutant, particularly *MEP1*, under low-concentration ammonium conditions. Interestingly, the Δ*areA* mutant showed reduced PKA activity and Gpmk1 phosphorylation. Both the cAMP-PKA and Gpmk1 MAPK pathways are known to be important for plant infection,

sexual reproduction and DON production in *F. graminearum* (Hu *et al*., 2014; Jenczmionka *et al*., 2003), which may be related to some of the phenotypes of the Δ*areA* mutant. RAS proteins are known to function upstream from the cAMP signalling and MAPK pathways in *S. cerevisiae*, *M. oryzae* and other fungi (Li *et al*., 2012; Zhao and Xu, 2007). In *C. albicans*, the C-terminal region of Mep2 has been implicated in association with Ras proteins. In *F. graminearum*, deletion of *AREA* significantly reduced the expression of all three *MEP* genes, which may, in turn, affect RAS signalling and the downstream cAMP-PKA and MAPK pathways. Therefore, it will be important to determine the relationship between MEPs and RAS signalling in *F. graminearum* in the future.

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 were routinely cultured at 25 °C and preserved in 15% glycerol at –80 °C. The split-marker approach was used to generate the gene replacement constructs for the *AREA* gene (Fig. S1). Putative knockout mutants were identified by PCR and further confirmed by Southern blot hybridizations. Cultures grown on CM, potato dextrose agar (PDA) and minimal medium (MM) were used for measurement of the growth rate or colony morphology (Hou *et al*., 2002). Conidiation was assayed in 5-day-old liquid CarboxyMethylCellulose (CMC) cultures as described previously (Ding *et al*., 2009; Zhou *et al*., 2010). Protoplasts were prepared and used for transformation as described previously (Hou *et al*., 2002). Hygromycin B (CalBiochem, La Jolla, CA, USA) and geneticin (Sigma-Aldrich, St. Louis, MO, USA) were added to final concentrations of 300 and 400 μg/mL, respectively, to the regeneration medium for transformant selection.

Generation of the AreA-GFP fusion construct and transformant

For complementation assays, a 4.3-kb fragment containing the entire *AREA* gene and 1.5-kb promoter region was amplified and cloned into pFL2 (Zhou *et al*., 2011a) by the yeast *in vivo* homologous recombination approach as described previously (Bruno *et al*., 2004). The resulting AreA-GFP construct was transformed into the *areA* deletion mutant RH12. Transformants expressing the AreA-GFP construct were verified by PCR. GFP signals in conidia and germlings were observed with an Olympus BX51 epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

Generation of the *AREA*$^{\Delta NLS1}$-, *AREA*$^{\Delta NLS2}$-, *AREA*$^{\Delta NLS3}$-, *AREA*$^{\Delta 657-658}$-, *AREA*$^{\Delta 874}$- and *AREA*^{S874A}-GFP fusion constructs and transformants

To generate the *AREA*$^{\Delta NLS1}$-GFP fusion construct by the yeast gap repair approach (Bruno *et al*., 2004), PCR products amplified with the primer pairs AREA-COMP-F/NLS1-R and NLS1-F/AREA-COMP-R were co-transformed into the yeast strain XK1-25 with *Xho*I-digested plasmid pFL2 (Zhou *et al*., 2011a). Plasmids rescued from the Trp$^+$ yeast transformants were then sequenced to confirm the *AREA*$^{\Delta NLS1}$-GFP construct. Similar strategies were used to generate the *AREA*$^{\Delta NLS2}$-, *AREA*$^{\Delta NLS3}$-, *AREA*$^{\Delta 657-658}$-, *AREA*$^{\Delta 874}$- and *AREA*^{S874A}-GFP fusion constructs by yeast gap repair (Bruno *et al*., 2004) with the primers listed in Table S1 (see Supporting Information). All these mutant alleles of *AREA* were confirmed by sequencing analysis and transformed into the Δ*areA* deletion mutant RH12. The resulting transformants were screened by PCR to confirm the presence of transforming constructs and examined for GFP signals by epifluorescence microscopy.

Sexual reproduction assays

To assay defects in sexual reproduction with the *areA* mutant and complementation strains, 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 previously (Li *et al*., 2011; Luo *et al*., 2014). Perithecium formation, cirrhi production and the formation of asci and ascospores were examined 2–3 weeks after induction (Zheng *et al*., 2013).

Plant infection and DON production assays

Conidia freshly harvested from CMC cultures were re-suspended to 10^5 spores/mL in sterile water and used for plant infection assays. Flowering wheat heads of cultivars Norm and Xiaoyan22 were inoculated with 10 μL of conidium suspensions at the fifth spikelet from the base of the inflorescence (Gale *et al*., 2007). Spikelets with typical head blight symptoms on each inoculated head were counted at 14 days post-inoculation (dpi). Results from three biological replicates with over 10 inoculated wheat heads each were used to estimate the disease index (Chen *et al*., 2014; Hou *et al*., 2002). Stalks of 8-week-old corn plants of cv. Pioneer 2375 were inoculated with tooth-picks dipped in conidium suspensions and examined for stalk rot symptoms as described previously (Zhou *et al*., 2010). For rice grain cultures (Seo *et al*., 1996), DON and ergosterol production were assayed as described previously (Bluhm *et al*., 2007; Seong *et al*., 2009). For each strain, DON production was also assayed with liquid-inducing cultures containing 5 mM $NaNO_3$ or arginine (Gardiner *et al*., 2009a).

qRT-PCR analysis

RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from vegetative hyphae harvested from 24 h YEPD cultures. For qRT-PCR analysis, first-strand cDNA was synthesized with the Fermentas 1st cDNA synthesis kit (Hanover, MD, USA) following the instructions provided by the manufacturer. The β-tubulin gene FGSG_06611 of *F. graminearum* was used as the internal control (Bluhm *et al*., 2007). Relative expression levels of each gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data from three biological replicates were used to calculate the mean and standard deviation.

Yeast two-hybrid assays

The Matchmaker yeast two-hybrid system (Clontech, Mountain View, CA, USA) was used to assay the interactions of AreA with Tir6 and Tri10. The

open reading frame (ORF) of the *AREA* gene was amplified from first-strand cDNA of PH-1 and cloned into pGBK7 (Clontech) as the bait construct. For the *TRI6* and *TRI10* genes, their ORFs were cloned into pGADT7 as the prey constructs. The resulting bait and prey vectors were transformed into the yeast strain AH109 (Clontech). Growth on synthetic dropout medium lacking tryptophan, leucine, and histidine (SD-Trp-Leu-His) medium and galactosidase activity were assayed as described previously (Zhou *et al*., 2011b). The positive and negative controls were provided in the Matchmaker Library Construction and Screening Kits (Clontech).

Co-immunoprecipitation assays

The *TRI6* and *TRI10* genes were amplified and cloned into pDL2 by the yeast gap repair approach (Bruno *et al*., 2004) to generate the GFP fusion constructs. Similar approaches were used to generate the 3 × FLAG fusion constructs for the *AREA* gene with the pFL7 vector (Zhou *et al*., 2011a). The resulting fusion constructs were verified by sequencing analysis and transformed in pairs into protoplasts of PH-1. Transformants expressing both GFP and FLAG fusion constructs were confirmed by Western blot analysis. For co-immunoprecipitation assays, total proteins were isolated (Bruno *et al*., 2004) and incubated with the anti-FLAG M2 beads as described previously (Liu *et al*., 2011). Western blots of proteins eluted from anti-FLAG beads were detected with monoclonal anti-GFP and anti-FLAG antibodies (Roche, Indianapolis, IN, USA).

Assays for Threonine-Glutamine-Tyrosine (TEY) phosphorylation and PKA activities

Total proteins were isolated from hyphae harvested from 48-h YEPD cultures and separated on 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bruno *et al*., 2004; Ding *et al*., 2010). After transfer onto nitrocellulose membranes, the phosphorylation of Mgv1 and Gpmk1 was detected with the PhophoPlus p44/42 antibody kit (Cell Signaling Technology, Danvers, MA, USA) as described previously (Liu *et al*., 2011). The expression level of Gpmk1 was detected with the anti-Pmk1 antibody generated in a previous study (Bruno *et al*., 2004). PKA activities were assayed with the PepTag nonradioactive PKA assay kit (Promega, Madison, WI, USA) as described previously (Adachi and Hamer, 1998; Nishimura *et al*., 2003).

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SUPPORTING INFORMATION

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

Fig. S1 The *AREA* gene replacement construct and mutant. (A) Generation of the *AREA* gene replacement construct. 1F, 2R, 3F and 4R are primers used to amplify the flanking sequences. *hph*, hygromycin phosphotransferase gene. (B) Southern blots of the wild-type strain PH-1 and Δ*areA* transformants (M1, M2 and M3) were hybridized with probe 1 amplified with primers *AREA*/5F and *AREA*/6R and probe 2 amplified with H852 and H850. (C) Three-day-old complete medium (CM) and minimal medium (MM) cultures of PH-1, Δ*areA* mutant RH12 and complementation strain (AC6). (D) Conidia of the same set of strains incubated in yeast extract peptone dextrose (YEPD) for 6 h. Bar, 20 μm.

Fig. S2 Defects of the Δ*areA* mutant in plant infection and self-crosses. (A) Flowering wheat heads were drop inoculated with conidia from the wild-type strain PH-1, Δ*areA* mutant and complementation strain (AC6). Black dots mark the inoculated spikelets. (B) Corn stalks were inoculated with the same set of strains. Photographs were taken at 14 days post-inoculation (dpi). (C) Perithecia and cirrhi produced by PH-1, Δ*areA* mutant and AC6. Bar, 1 mm. (D) Two-week-old perithecia were cracked open and examined for asci and ascospores with the same set of strains. Bar, 50 μm.

Fig. S3 Functional characterization of two putative nuclear localization signal (NLS) sequences. Germlings of the Δ*areA/AREA*$^{\Delta NLS1}$-GFP transformants (RL10 and RL11) and Δ*areA/AREA*$^{\Delta NLS2}$-GFP transformants (RL12 and RL13) were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by differential interference contrast (DIC) and epifluorescence microscopy. GFP, green fluorescent protein. Bar, 10 μm.

Fig. S4 Putative AreA-binding sites in promoter region (within 1 kb) of the three *MEP* genes. White triangle, H (A, C or T)/GATA/R (A or G); black triangle, Y (T, G or A)/TATC/D (T or C).

Table S1 Polymerase chain reaction (PCR) primers used in this study.

Table S2 Deoxynivalenol (DON) production in PH-1 and its transformants expressing different mutant alleles of *AREA*.