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Functional Roles of a Putative B' Delta Regulatory Subunit and a Catalytic Subunit of Protein Phosphatase 2A in the Cereal Pathogen *Fusarium graminearum*

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Protein phosphatase 2A (PP2A), a family of serine/threonine protein phosphatases, plays an important role in balancing the phosphorylation status of cellular proteins for regulating diverse biological functions in eukaryotic organisms. Despite intensive studies in mammals, limited information on its role is available in filamentous fungi. Here, we investigated the functional roles of genes for a putative B' delta regulatory subunit (*FgPP2AR*) and a catalytic subunit (*FgPP2AC*) of PP2A in a filamentous ascomycete, *Fusarium graminearum*. Molecular characterization of an insertional mutant of this plant pathogenic fungus allowed us to identify the roles of *FgPP2AR*. Targeted gene replacement and complementation analyses demonstrated that the deletion of *FgPP2AR*, which was constitutively expressed in all growth stages, caused drastic changes in hyphal growth, conidia morphology/germination, gene expression for mycotoxin production, sexual development and pathogenicity. In particular, overproduction of aberrant cylindrical-shaped conidia is suggestive of arthroconidial induction in the $\Delta FgPP2AR$ strain, which has never been described in *F. graminearum*. In contrast, the $\Delta FgPP2AC$ strain was not significantly different from its wild-type progenitor in conidiation, trichothecene gene expression, and pathogenicity; however, it showed reduced hyphal growth and no perithecial formation. The double-deletion $\Delta FgPP2AR;\Delta FgPP2AC$ strain had more severe defects than single-deletion strains in all examined phenotypes. Taken together, our results indicate that both the putative regulatory and catalytic subunits of PP2A are involved in various cellular processes for fungal development in *F. graminearum*.

Keywords : aberrant conidia morphology, B regulatory subunit, catalytic subunit, *Fusarium graminearum*, protein phosphatase 2A

Molecular regulation of many biological functions in eukaryotic organisms is mediated by protein posttranslational modifications. Protein phosphorylation is one of the major mechanisms in this regulatory system. In particular, reversible phosphorylation of cellular proteins in response to external or intracellular stimuli is an essential step for signal transmission to specific transcription factors that regulate downstream genes in a specific signal transduction cascade (Liu et al., 2000; Luan, 2003). The phosphorylation status of target proteins is modulated by the reversible activities of protein kinases and protein phosphatases. Serine/threonine protein phosphatases (PPs), which catalyze the dephosphorylation of proteins, comprise two different structural families based on biochemical characteristics: type 1 (PP1) and type 2 (PP2). PP2s are subsequently divided into three groups (2A, 2B, and 2C). Protein phosphatase 2A (PP2A) holoenzyme, a member of the PP2 family, consists of a core dimer comprising a catalytic C subunit (PP2AC) and a scaffolding A subunit, which can be complexed with a third variable regulatory B subunit (PP2AB) to form a heterotrimer (Mumby, 2007). The variable B subunits, which can determine the substrate specificity, catalytic activity, and cellular localization of PP2A, are grouped into four unrelated families, namely B (PR55), B' (B56/PR61), B'' (PR48/59/72/130) and B''' (PR93/110) based on structural similarities. Among the B regulatory subunits, the B' subunit is the most diverse, consisting of five isoforms (alpha, beta, gamma, delta, and epsilon) (McCright et al., 1996; Muneer et al., 2002; Sontag, 2001). The diversity of possible combinations of these subunits allows the PP2A holoenzyme to play roles in the regulation of a wide range of cellular and metabolic processes in a variety of organisms (Gallego and Virshup, 2005; Lechward et al., 2001). Despite intensive investigations in human and model organisms, limited information on the role of PP2A is available in filamentous fungi.

Fusarium graminearum (teleomorph: *Gibberella zeae*) is a filamentous ascomycetous fungus that threatens both plant and animal health not only by causing disease but also

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by producing mycotoxins in cereal plants (Desjardins, 2006; McMullen et al., 1997). To explore the mechanisms underlying the development of disease caused by this fungus, we have generated a collection of insertional mutants from a representative strain of *F. graminearum* using the restriction enzyme-mediated integration (REMI) procedure (Han et al., 2007; Seo et al., 2007). A screening of REMI transformants resulted in the identification of a mutant that carried a mutation in the gene encoding a putative B-regulatory subunit (B' delta isoform) of PP2A, whose function had not been elucidated in filamentous fungi. Because many fungal traits involved in disease development such as pathogenicity, sexual development, and mycotoxin production are known to be controlled by signal transduction cascades (Yu et al., 2008), this mutant is useful for dissecting the regulatory pathways mediated by PP2A for these traits in *F. graminearum*. Therefore, the objective of

this study was to determine the functional roles of a putative PP2A holoenzyme comprising the variable B regulatory subunit and a putative catalytic subunit in *F. graminearum* using molecular genetic approaches.

Materials and Methods

Fungal strains and cultures. The wild-type (WT) *F. graminearum* strain used in this study, Z03643, is self-fertile and belongs to lineage 7 of the *F. graminearum* species complex (Kim et al., 2011; O'Donnell et al., 2000). The Z43R2629 strain was generated through REMI mutagenesis of Z03643 using the pIGPAPA DNA digested with *Kpn*I (Seo et al., 2007). Fungal strains from 25% glycerol stock cultures stored at -80°C were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). For vegetative growth and conidiation, the strains

Table 1. Primers used in this study

Position ^a	Name	Nucleotide sequence ^b (5'-3')	Amplified region
a	phos5for	GAGCGCATGTTTTCTAAGG	
b	phos5revtail	<u>GCACAGGTACACTTGTTTAGAGTCCAAAATCGAAGCATCAA</u>	5' flank of <i>FgPP2AR</i>
c	phos5nest	GATGAAGGAGAGGAAAAGAAATGGTGGTAA	
d	phos3fortail	<u>CCTTCAATATCATCTTCTGTCGGTGTCGTGCGGAGGGGCGGTTTA</u>	
e	phos3rev	TACTTCATGGCCTGTGCTTTAGA	3' flank of <i>FgPP2AR</i>
f	phos3nest	TGTGAAGCATTGCGTCGAGTAAAGGTG	
g	Ppe1-5for	GCCATCCTTCACGTTCTTTGCCTTTTTC	
h	Ppe1-5revtail	<u>ACCTCCACTAGCTCCAGCCAAGGATCAAGCATGCCAGTCG</u>	5' flank of <i>FgPP2AC</i>
i	Ppe1-5nest	CTTCGACTGTTTCCTTTGGTGGTGTGG	
j	Ppe1-3fortail	<u>TATGAAAATTCCGTCACCAGCCTGTAGAATAGCATAGGGTCATA</u>	
k	Ppe1-3rev	TTTGTAAGCTTAATGTTGAG	3' flank of <i>FgPP2AC</i>
l	Ppe1-3nest	TCGTGGGAAGAAAGCCGCAGTTGATG	
m	PHO-tailgfp	<u>CAGCTCCTCGCCCTTGCTCACCATCTTGTCGGACAACAGTGTGTCG</u>	a promoter region of <i>FgPP2AR</i>
n	GFP/p5	ATGGTGAGCAAGGGCGAGGAGCTGT	<i>GFP</i>
o	GFP/P6	CTTGTAACAGCTCGTCCATGCCGTGAG	<i>GFP</i>
p	HygB-5for	CTTGGCTGGAGCTAGTGGAGGT	<i>hygB</i>
q	HygB-3rev	GGCTGGTGACGGAATTTTCATA	<i>hygB</i>
r	Gen/for	CTCTAAACAAGTGACCTGTGC	<i>gen</i>
s	Gen/rev	CGACAGAAGATGATATTGAAGG	<i>gen</i>
	3536for	AGCCAGCTTATCGCCCTTCC	
	3536rev	AGCCTTTGGTGCCGACTTCTTG	for qRT-PCR of <i>TRI6</i>
	8081for	GTGCATGTTCTGGCCGACCTTGTT	
	8081rev	GTGCATGTTCTGGCCGACCTTGTT	for qRT-PCR of FGSG_08081.3

^aThe positions of these primers are indicated in Fig. 2.

^bThe underlined sequences are complementary to the genes for selectable markers (*hygB*, *gen*, or *GFP*).

were grown in either PDA, complete medium (CM; Leslie and Summerell, 2006) or YMA medium (Semighini et al., 2008). To induce sexual development, fungal strains were grown on carrot agar plates as described previously (Kim et al., 2008; Leslie and Summerell, 2006). For total RNA extraction under trichothecene-inducing conditions, a fungal strain was inoculated into minimal medium amended with agmatine at a final concentration of 1×10^4 conidia/mL and incubated as previously described (Gardiner et al., 2009).

Nucleic acid manipulations and PCR. General procedures for extraction of fungal genomic DNA or total RNA, restriction endonuclease digestion, agarose gel electrophoresis, gel blotting, ^{32}P probe labeling, and hybridization were performed as described previously (Kim et al., 2008; Sambrook and Russell, 2001). The polymerase chain reaction (PCR) primers (Table 1) used in this study were synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corp., Chungwon, Korea). Quantitative real-time PCR was performed as previously described (Kim and Yun, 2011) using total RNAs isolated from mycelia grown in the agmatine-amended liquid medium at two time points. Gene expression was measured in two independent samples from each time point using three technical replicates. Gene expression of *EF1A* (FGSG_08811.3) was used as endogenous controls for data normalization. The amount of each gene transcript from a 4-day-old sample was used as a reference for comparison.

Vector construction. A fungal transforming DNA construct for targeted gene deletion was created using the double-joint PCR method, as described previously (Lee et al., 2009; Yu et al., 2004). DNA fragments corresponding to the 5' and 3' flanking regions of the *FgPP2AR* ORF were amplified from the genomic DNA of Z03643 using the a/b and d/e primer pairs (Table 1 and Fig. 3 for the position of primers), respectively, and mixed with the *gen* gene cassette amplified with primers r and s (Table 1) from the vector pII99 (Namiki et al., 2001). A second round of PCR using the nested c and f primer pair (Table 1) resulted in a 4.1-kb fragment of the final fusion PCR product. For $\Delta FgPP2AC$, the 5' and 3' flanking regions of the *FgPP2A* ORF were amplified using the g/h and j/k primer pairs (Table 1), respectively, and mixed together with the *hygB* gene cassette from the pBCATPH vector (Yun, 1998). The second round of PCR using the nested i and l primer pair (Table 1) generated the final DNA construct for gene deletion. For double deletion of *FgPP2AR* and *FgPP2AC*, the $\Delta FgPP2AC$ strain was transformed with the DNA construct for $\Delta FgPP2AR$ as described above. For molecular complementation, an intact copy of *FgPP2AR* or *FgPP2AC* gene fragments (including its native promoter and terminator

regions) was amplified from Z03643 using the c/f and i/l primer pairs, respectively, cloned into a pGEM-T Easy vector (Promega), and added to the $\Delta FgPP2AR$ protoplasts along with the pBCATPH vector carrying *hygB*, or to the $\Delta FgPP2AC$ protoplasts along with pII99 carrying *gen*.

Fungal transformation and virulence test. All PEG-mediated fungal transformation steps using the DNA constructs described above were performed as described previously (Kim et al., 2008; Kim et al., 2011). The virulence of the fungal strains was determined on young ears of corn or on spikelets of barley as described previously (Han et al., 2007; Lee et al., 2009).

Results

Phenotypes and molecular characterization of the REMI mutant Z43R2629. A REMI mutant of *F. graminearum*, designated Z43R2629, showed pleiotropic phenotypes in several traits such as hyphal growth, pigmentation, sexual development, and pathogenicity on corn ears (Fig. 1). Compared to its WT progenitor Z03643, the radial hyphal growth of Z43R2629 was reduced to ~30% of that of Z03643, with fewer aerial mycelia and no red pigmentation when grown on PDA or carrot agar (Fig. 1). Under the sexual induction condition, Z43R2629 formed no perithecia even 6 weeks after removing the aerial mycelia that had already grown on carrot agar for 1 week; the WT strain usually began to form fertile perithecia 4 days after sexual induction (Fig. 1). In addition, the ability of Z43R2629 to cause disease symptoms was almost abolished on young corn ears, whereas inoculation with Z03643 resulted in a large area of necrosis around the inoculation points (Fig. 1).

Fig. 1. Comparison of phenotypes in the *Fusarium graminearum* WT Z03643 strain (A) and Z43R2629 strain (B). Growth of aerial mycelia on carrot agar, perithecia formation on carrot agar, and symptoms on corn ears are shown in the left, center, and right panels, respectively.

Fig. 2. Molecular analyses of the vector insertions in the Z43R2629 genome. (A) A blot of genomic DNA of Z43R2629 digested with *KpnI* (lane 1) and *BglII* (lane 2) was hybridized with pIGPAPA. The sizes of lambda DNA standards (in kilobases) are indicated to the left of the blot. (B) Molecular structures of the vector insertion site in Z43R2629. The open reading frames (ORFs) near the vector insertion point are indicated by the filled arrow. The dashed lines represent the genomic regions that are not present at the vector insertion points in Z43R2629, probably due to a rearrangement between chromosomes 1 and 3.

The vector insertion point in the Z43R2629 genome was identified using DNA gel blot analysis (Fig. 2). In genomic DNA digested with *KpnI*, the enzyme used to linearize the vector DNA (pIGPAPA) in the REMI procedure, the entire sequence of pIGPAPA hybridized to a single ~5.9-kb fragment the same size as pIGPAPA (Fig. 2A), indicating a single vector insertion in the Z43R2629 genome, typical of a real REMI event. When the genomic DNA was digested with *BglII*, for which the vector has no recognition site, a single hybridizing band of ~8.4 kb appeared (Fig. 2A). Nucleotide sequencing of the 8.4-kb *BglII* fragment recovered using a plasmid rescue procedure revealed that it contains 1.3 kb of genomic DNA to the left side and 7.1 kb to the right side of the vector. However, BLAST searches of the *F. graminearum* genome database (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) revealed that the immediate left and right flanking regions could not be connected into a continuous open reading frame (ORF). Instead, the left flanking sequence of the vector started at a *KpnI* site 230 bp downstream of the putative stop codon of an ORF (annotated as FGSG_01142 in the fungal genome) located on chromosome 1, while the right flanking sequence started at a *KpnI* site within an ORF (FGSG_05894) of 655 amino acids (interrupted by four putative introns) located on chromosome 3 (Fig. 2B). The entire deduced sequence of FGSG_05894, designated *FgPP2AR*, had high similarity to the putative B' (also known as B56)-delta regulatory subunit of protein phosphatase 2A. This vector insertion pattern suggests that chromosomal rearrangement occurred during REMI mutagenesis in the genome of Z43R2629.

Targeted deletions of *FgPP2AR* and *FgPP2AC*. To confirm that the disruption of *FgPP2AR* was a direct cause of pleiotropic changes in Z43R2629, the genomic copy of *FgPP2AR* was deleted from the genome of *F. graminearum* Z03643 strain using a targeted gene replacement strategy (Fig. 3). Double crossover integration of the fusion PCR product carrying a *gen* cassette fused to the 5' (1.0 kb) and 3' (1.0 kb) regions flanking *FgPP2AR* into the fungal genome resulted in the deletion of *FgPP2AR* (designated $\Delta FgPP2AR$) (Fig. 3A). Targeted gene deletion was verified by DNA gel blot analysis (Fig. 3A). When genomic DNA from the $\Delta FgPP2AR$ strains was digested with *NcoI*, a single 4.7-kb hybridizing band was observed, instead of the 6.9-kb band that hybridized in Z03643, confirming that the entire *FgPP2AR* ORF had been deleted and replaced with *gen*, as expected in the $\Delta FgPP2AR$ strains. All of the examined $\Delta FgPP2AR$ strains exhibited the same pleiotropic changes on PDA and carrot agar as did the REMI mutant Z43R2629 (Figs. 1 and 4).

To determine the function of a putative catalytic subunit of the protein phosphatase 2A (PP2A) in *F. graminearum*, the *FgPP2AC* gene was deleted from the Z03643 genome, as described above. *FgPP2AC*, annotated as FGSG_01464.3 in the genome database, is an ortholog of the *F. verticillioides* *CPP1* encoding a putative PP2A catalytic subunit (Choi and Shim, 2008). The entire 401-amino acid ORF of *FgPP2AC* was replaced with a *hygB* cassette by double cross-over between a fusion PCR construct and the fungal genome, which was verified by DNA gel blot hybridization (Fig. 3B). The *BglII*-digested genomic DNA from the $\Delta FgPP2AC$ strains showed a single 17.0-kb hybridizing

Fig. 3. Targeted deletions of *FgPP2AR* (A), *FgPP2AC* (B), and both genes (C) from the genome of *F. graminearum* WT strain Z03643, and genetic complementation. (Left in each panel) Deletion and complementation strategies. WT: genomic DNA of Z03643, $\Delta FgPP2AR$: the Z03643 strain with *FgPP2AR* deleted, $\Delta FgPP2AC$: the strain with *FgPP2AC* deleted, $\Delta FgPP2AR$; $\Delta FgPP2AC$: the strains carrying the double gene deletion, *CoFgPP2AR*: the $\Delta FgPP2AR$ carrying an intact copy of *FgPP2AR* ORF, and *CoFgPP2AC*: the $\Delta FgPP2AC$ carrying an intact copy of *FgPP2AC* ORF. The transcription direction of each gene is indicated by the filled arrow. The positions of primers used in the construction of transforming vectors (see the Materials and Methods section) are indicated by arrows with the primer names (see Table 1). (Right in each panel) Gel blots of *Nco*I-digested genomic DNA (A and C) and *Bgl*II-digested genomic DNA (B) from transgenic strains, probed with the DNA fragments amplified with primer pairs d/f and j/k, respectively. Lanes in A and B: 1, Z03643; 2–3, the gene deletion strains; 4–5, the complemented strains. Lanes in C: 1, the $\Delta FgPP2AC$ strain; 2, the double gene deletion strain.

band, instead of the 16.0-kb band in Z03643 (Fig. 3B). To construct the transgenic *F. graminearum* strain lacking both *FgPP2AR* and *FgPP2AC* ($\Delta FgPP2AR$; $\Delta FgPP2AC$), the genomic copy of *FgPP2AR* was deleted from the genome of a $\Delta FgPP2AC$ strain using the same strategy as described for the deletion of *FgPP2AR* from Z03643 (Fig. 3C); the double gene deletion was confirmed by DNA gel blot analysis (Fig. 3C).

Genetic complementation of $\Delta FgPP2AR$ and $\Delta FgPP2AC$.

For further confirmation of the gene function in *F. graminearum*, an intact copy of *FgPP2AR* or *FgPP2AC* was introduced into the genome of a $\Delta FgPP2AR$ or a $\Delta FgPP2AC$ strain, respectively. A 4.0-kb *FgPP2AR* DNA fragment including 5' (1.0 kb) and 3' (1.0 kb) flanking sequences was amplified from the genomic DNA of Z03643 using the c and f primer pair (Table 1) and directly added into protoplasts of the $\Delta FgPP2AR$ strain, along with pBCATPH carrying *hygB* as a fungal selectable marker

(Fig. 3A). A total of 8 of 18 *hygB*^R transformants were no longer resistant to geneticin, suggesting the *FgPP2AR* gene was integrated into its original genomic locus of the $\Delta FgPP2AR$ strain by double cross-over, as confirmed by DNA gel blot analysis (Fig. 3A). Similarly, the deletion of *FgPP2AC* was successfully complemented with *FgPP2AC* using a co-transformation procedure with pII99 carrying *gen* (Fig. 3B).

Aberrant morphology caused by the gene deletions. The hyphal growth pattern of the $\Delta FgPP2AR$ strain was similar to that of the REMI mutant Z43R2629. Compared to the WT Z03643 strain, it produced less aerial mycelia with reduced radial growth and no red pigmentation on carrot agar (Figs. 1 and 4). The $\Delta FgPP2AC$ strain also showed a reduced radial growth similar to the $\Delta FgPP2AR$ strain, but its mycelia were more heavily pigmented with purple/red (Fig. 4). The $\Delta FgPP2AR$; $\Delta FgPP2AC$ strains exhibited less than 10% of the WT level of radial growth with no

Fig. 4. Mycelial growth (upper panel) and perithecia formation (lower panel) of the transgenic *F. graminearum* strains generated in this study on carrot agar. The names and descriptions of each strain are provided in the legend of Fig. 3.

Fig. 5. Hyphal growth (upper panels) and conidia morphology of the gene deletion strains grown on YMA medium. (A) Z03643, (B) $\Delta FgPP2AR$ strain, (C) $\Delta FgPP2AC$ strain, (D) $\Delta FgPP2AR; \Delta FgPP2AC$ strain. Scale bar = 100 μ m.

pigmentation (Fig. 4). In hyphal morphology, both the $\Delta FgPP2AR$ and $\Delta FgPP2AC$ strains were not significantly different from the WT strain, but the double deletion strain showed thicker and swollen hyphae (Fig. 5).

The deletion of *FgPP2AR* led to an aberrant conidial shape and germination in *F. graminearum* (Figs. 5 and 6). The 3–5 septate macroconidia formed on aerial mycelia in Z03643 were hyaline, slightly curved, and tapering at both ends with a foot-celled base, typical of the conidia morphology of *F. graminearum* (Fig. A). In contrast, the $\Delta FgPP2AR$ strain produced no typical macroconidia, instead forming a long cylindrical-shaped conidia with a rounded apex and a truncate, rounded base with variable sizes (Fig. 5B). The double-deletion strain produced neither the typical falcate nor truncate conidia observed in the Z03643 and $\Delta FgPP2AR$ strains, respectively. Instead, its hyphae became thicker than those from these two strains, and the hyphal tips were swollen from which minor constriction chains were probably made. In addition, it produced hyphal frag-

ments with swollen intercalary and terminal cells (or minor constructions; Fig. 5D). However, it is unclear whether these aberrant structures resulted from the ultimate disarticulation of the hyphal chains with minor constrictions. However, the morphology of conidia formed in the $\Delta FgPP2AC$ strain was not different from that in Z03643 (Fig. 5C). Within 3 h of incubation, the conidia from both the Z03643 and $\Delta FgPP2AC$ strains were swollen and began to form germination tubes from terminal cells, intercalary cells, or both at 5 h (Fig. 6A and C). However, the truncate conidia of the $\Delta FgPP2AR$ strain formed the germ tube at 7–9 h from terminal regions only (Fig. 6B). In the double-deletion strain, germ tubes started to emerge from swollen cells in both ends of arthroconidia-like structures at 3–5 h, but elongated more slowly compared to the case of Z03643 (Fig. 6D). These aberrant morphologies were restored to the WT level when an intact copy of *FgPP2AR* or *FgPP2AC* was introduced into the $\Delta FgPP2AR$ or $\Delta FgPP2AC$ strain, respectively (data not shown).

Fig. 6. Conidial germination of the gene deletion strains using complete medium (CM). (A) Z03643, (B) $\Delta FgPP2AR$ strain, (C) $\Delta FgPP2AC$ strain, (D) $\Delta FgPP2AR$; $\Delta FgPP2AC$ strain. Times elapsed (in h) after inoculation into CM are indicated above the panels. Scale bar = 100 μ m.

Fig. 7. Pathogenicity of the gene deletion strains on barley heads.

Defects in sexual development and virulence. The capacity of the $\Delta FgPP2AR$ strain for sexual reproduction was completely impaired as described above in the REMI mutant Z43R2629 (Fig. 4). In addition, the $\Delta FgPP2AC$ and double-deletion strains were unable to form perithecia even after more than 6 weeks; the WT Z03643 strain formed abundant perithecia containing ascospores on carrot agar within 2 weeks (Fig. 4).

All of the sexual defects in these gene deletion strains were restored to the WT level when genetically complemented in strains $\Delta FgPP2AR::FgPP2AR$ and $\Delta FgPP2AC::FgPP2AC$ (Fig. 4). In addition, we examined the pathogenic development of the gene deletion strains on a host plant. Inoculation with the $\Delta FgPP2AR$ strain had almost no effect on barley heads, resulting in no development of

disease symptoms (Fig. 7). The abolished virulence was also evident in the double-deletion strain (Fig. 7). Unlike these strains, the $\Delta FgPP2AC$ strain caused necrotic spots on approximately 70–80% of spikelets of each barley head examined by 10 days after inoculation, and these symptoms developed into a typical head blight as severe as those caused by the Z03643 strain (Fig. 7). The WT level symptoms were observed when each genetically complemented strain described above was inoculated (Fig. 7).

Changes in expression of secondary metabolite genes. To determine if *F. graminearum* protein phosphatase 2A is involved in expression of genes responsible for biosyntheses of several toxic metabolites, we quantified the relative expression level of two genes (*TRI6* and *FGSG_08081.3*),

Fig. 8. The expression pattern of *TRI6* and *FGSG_08081.3* in agmatine-amended liquid medium for the production of trichothecenes and butenolide determined using quantitative real-time PCR. The numbers on the y-axis represent the relative amount of each gene transcript. The numbers on the x-axis indicate the names of deletion strains grown in the liquid medium for 4 and 8 days. Error bars represent standard error.

which were involved in the production of the mycotoxins trichothecene and butenolides in the agmatine-amended medium. The expression level of *TRI6* was significantly ($p < 0.05$) reduced in both the $\Delta FgPP2AR$ and the double-deletion strains, whereas expression in the $\Delta FgPP2AC$ strain was not different compared to those in the WT strain when grown in agmatine-amended liquid medium, an

inducing condition for the production of trichothecenes and butenolide. The gene expression level from the double deletion mutant at day 4 was not available due to insufficient mycelia growth at that time point (Fig. 8). Unlike the case of *TRI6*, the expression of *FGSG_08081.3* was dramatically down-regulated in all of the deletion strains at day 8 in the agmatine medium (Fig. 8).

Fig. 9. Expression of GFP under control of a putative promoter region of *FgPP2AR*. (A) Scheme for introduction of the *GFP* gene into the fungal genome. The fusion PCR product of the promoter region and *GFP* gene, amplified with primer pairs m/f and d/n (Table 1), was cloned into pGEMT, and added to the Z03643 strain along with pBCATPH. (B) DNA gel blot of hygromycin B-resistant transformants of Z03643 probed with the *GFP* gene. Lanes 1, Z03643; 2–3, hyg^R transformants. (C) Observation of mycelia, conidia, and ascospores under light (upper panels) and fluorescence (lower panels) microscopes.

Expression of green fluorescence protein under a putative promoter region of *FgPP2AR*. To determine the expression pattern of *FgPP2AR* during the entire growth stages of *F. graminearum*, plasmid DNA carrying the GFP gene under the control of a 1.0 kb putative promoter region of *FgPP2AR* was introduced into the Z03643 strain along with pBCATPH (Fig. 9A). All of the hygromycin B-resistant transformants carrying the GFP gene construct, confirmed by DNA gel blot analysis (Fig. 9B), expressed GFP in the stages of mycelia, conidia, and ascospores at all examined time points, confirming that *FgPP2AR* was expressed constitutively in all of the cell types (Fig. 9C).

Discussion

Despite the chromosomal rearrangement at the vector insertion region in a REMI mutant Z43R2629, the molecular characterization of Z43R2629 allowed the identification of the functional roles of a putative delta isoform of B' (B56) regulatory subunit of PP2A (*FgPP2AR*) in *F. graminearum*, which had never been characterized in filamentous fungi. Although the B' regulatory subunit is the most diverse class among the three families (B, B', and B'') of the B-regulatory subunits, its functional roles have been intensively determined only in mammals (Bobulescu et al., 2010; Flegg et al., 2010; Hahn et al., 2010; Louis et al., 2011; Muneer et al., 2002). Specifically expressed in both embryonic cells and brain, it is involved in the dephosphorylation of proteins in various signaling pathways (McCright et al., 1996). The only B-regulatory subunit of PP2A whose functions have been analyzed in filamentous fungi is the B class subunit encoded by *rgb-1* and its homolog in *N. crassa* and *S. sclerotinia*, respectively (Erental et al., 2007; Yang et al., 2004; Yatzkan and Yarden, 1999). Among the pleiotropic changes caused by $\Delta FgPP2AR$, which were confirmed by gene deletion and complementation analyses, the phenotype of aberrant conidia morphology has never been described in the various *F. graminearum* mutants. The aberrant cylindrical-shaped conidia predominantly found in the $\Delta FgPP2AR$ strain, which lacks both the notched apex and classic *Fusarium* foot-celled base, seem to be similar to arthroconidia, and have never been observed in *F. graminearum*. This abnormal conidiation in the *F. graminearum* $\Delta FgPP2AR$ strain is comparable to that in the *N. crassa* *rgb-1^{RIIP}* strain (Yatzkan and Yarden, 1999). In WT *N. crassa*, cylindrical-shaped arthroconidia comprise roughly 1% of the total conidial mass (Springer and Yanofsky, 1989), whereas the *rgb-1* mutant shows overproduction of arthroconidia along with reduced normal macroconidiation (Yatzkan and Yarden, 1999). If the aberrant conidia in the $\Delta FgPP2AR$ are true arthroconidia, it is likely that *FgPP2AR* participates in the regulatory or signaling

pathway(s) required for normal conidiation in *F. graminearum*, which might be common in those controlled by RGB-1 in *N. crassa*. The induced arthroconidiation-like development was more evident when *FgPP2AC* encoding a putative catalytic subunit of PP2A was deleted in the genetic background of $\Delta FgPP2AR$. Because the single deletion of *FgPP2AC* caused no changes in conidia morphology, formation of frequent constrictions in hyphal chains and arthroconidia-like hyphal fragments in the double-deletion strain could be caused by an additive effect of $\Delta FgPP2AR$. In particular, the swollen and spherical structures formed at both ends of the hyphal fragments during germination are similar to those of arthroconidia in *N. crassa* (Springer and Yanofsky, 1989). However, this functional analogy between *FgPP2AR* and RGB-1 needs further consideration and experimental confirmation. The B-regulatory subunits encoded by these two genes belong to different classes (B' delta and B classes for *F. graminearum* and *N. crassa*, respectively). It remains unclear whether the enhanced or induced arthroconidiation would be a common phenotypic change in the fungal strains lacking B-regulatory subunits. In this respect, the fact that no changes in conidia morphology were observed in the *rgb-1* mutant of *S. sclerotinia* could argue against this hypothesis (Erental et al., 2007). It is also necessary to disrupt the *rgb-1* ortholog (FGSG_01369.3) in *F. graminearum* to determine whether it induces arthroconidiation as in the $\Delta FgPP2AR$ strain. Further microscopic analyses are necessary to elucidate the developmental processes involved in the production of the arthroconidia-like structure because hyphal fragmentations into arthroconidia were not clearly observed in the $\Delta FgPP2AR$ strain. Another possible scenario for the abnormal conidial morphology may be that conidium initials formed on a phialide were released without undergoing proper maturation for normal macroconidiation in the $\Delta FgPP2AR$ strain. Although further investigations are required to explore the mechanism underlying these defects, it is clear that *FgPP2AR* directs the holoenzyme of *FgPP2A* to control genes or proteins necessary for proper conidiogenesis in *F. graminearum*. Despite the uncertainties regarding conidiation, the defects in conidial morphology and germination (altered germination polarity) could be attributed, at least in part, to non-pathogenicity on host plants and self-sterility of the $\Delta FgPP2AR$ strain. It is also likely that $\Delta FgPP2AR$ may cause inappropriate expression of a variety of genes or proteins involved in sexual development and pathogenicity. Altered pigmentation of aerial mycelia and reduced transcription of genes for trichothecenes and butenolide in the $\Delta FgPP2AR$ strain also suggest that secondary metabolism is regulated by pathways controlled by *FgPP2AR* in *F. graminearum*. Constitutive GFP expression under the control of the *FgPP2AR*

promoter in mycelia, conidia, and ascospores indicates that *FgPP2AR* is ubiquitously expressed during all developmental stages (both asexual and sexual reproduction stages), which is comparable to the high expression of the B' delta subunit gene in human brain and embryo (McCright et al., 1996).

In contrast to *FgPP2AR*, the deletion of *FgPP2AC* encoding a putative catalytic subunit of PP2A has milder effects on the physiology of *F. graminearum*, although mycelial growth in the $\Delta FgPP2AC$ strain was reduced to the level similar to that in the $\Delta FgPP2AR$ strain. To date, the functional roles of two catalytic subunits of PP2A have been investigated in filamentous fungi: a homolog of *N. crassa pph-1* (Erental et al., 2007; Kosmidou et al., 2001; Yatzkan et al., 1998) and that of *N. crassa ppe-1* (Choi and Shim, 2008). The former was confirmed to be essential for fungal growth in *N. crassa*, *S. sclerotinia*, and *A. nidulans* because complete or partial gene inactivation led to lethality or slow hyphal growth. Due to their essential functions, the specific roles of these homologs could not be determined in fungal phenotypes except for hyphal growth. The latter, to which *FgPP2AC* in this study is orthologous (with 75% amino acid identity), is known to be involved in hyphal growth, conidial development/germination, hyphae morphology, and biosynthesis of a polyketide mycotoxin (fumonisin) in *F. verticillioides*, suggesting that this PP2AC is associated with multiple downstream signaling pathways in *F. verticillioides*. However, most phenotypic changes in the *F. verticillioides* strain lacking this ortholog (*CPP1*) were different from or not compatible to those in the *F. graminearum* $\Delta FgPP2AC$ strain, even though *CPP1* displayed high similarity to *FgPP2AC* (with 94% amino acid identity). For example, hyphal swelling and reduced conidia germination rates in the *F. verticillioides* $\Delta CPP1$ strain were not observed in the $\Delta FgPP2AC$ strain. The specific role of *CPP1* in probable negative regulation of the *FUM1* gene for fumonisin production in *F. verticillioides* cannot be directly compared to that of *FgPP2AC* because no functional homolog of *FUM1* is present in *F. graminearum*. Reduced expression of the gene for butenolide, but not for trichothecene in the $\Delta FgPP2AC$ strain, supports the specific but different role(s) of *FgPP2AC* in secondary metabolism compared to *F. verticillioides*. The phenotypes of pathogenicity and sexual development caused by $\Delta FgPP2AC$ cannot be compared to those by $\Delta CPP1$ because the effect of $\Delta CPP1$ in these traits was not investigated in *F. verticillioides* (Choi and Shim, 2008). These phenotype comparisons imply that *FgPP2AC* and its ortholog *CPP1* have overlapping functions in hyphal growth, but may participate in different regulatory pathways for other traits in two closely related fungal species. In addition, the milder effects of $\Delta FgPP2AC$ than $\Delta CPP1$ and even $\Delta FgPP2AR$ suggest that the function of *FgPP2AC* may be

redundant in *F. graminearum*. To confirm this, deletion of the gene (FGSG_09815.3) orthologous to the *N. crassa pph-1* from *F. graminearum* would be necessary. However, more severe changes in all of the phenotypes examined in the double gene deletion ($\Delta FgPP2AR$; $\Delta FgPP2AC$) strain, some of which were indistinguishable from the WT strain in the $\Delta FgPP2AC$ strain, indicates that a FgPP2A holoenzyme probably consisting of FgPP2AC and FgPP2AR is involved in controlling various regulatory and signal transduction pathways required for hyphal growth, conidiation, sexual development, mycotoxin production, and pathogenicity in *F. graminearum*. To explore the specific role(s) of these PP2A subunits in the physiology of *F. graminearum*, further investigations are necessary, such as the identification of downstream genes and target proteins for dephosphorylation, and confirmation of functional FgPP2A holoenzyme formation from FgPP2AC and FgPP2AR.

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