Protein phosphatase 2A regulatory subunits perform distinct functional roles in the maize pathogen *Fusarium verticillioides*

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SUMMARY

Fusarium verticillioides is a pathogen of maize causing ear rot and stalk rot. The fungus also produces fumonisins, a group of mycotoxins linked to disorders in animals and humans. A cluster of genes, designated *FUM* genes, plays a key role in the synthesis of fumonisins. However, our understanding of the regulatory mechanism of fumonisin biosynthesis is still incomplete. We have demonstrated previously that Cpp1, a protein phosphatase type 2A (PP2A) catalytic subunit, negatively regulates fumonisin production and is involved in cell shape maintenance. In general, three PP2A subunits, structural A, regulatory B and catalytic C, make up a heterotrimer complex to perform regulatory functions. Significantly, we identified two PP2A regulatory subunits in the *F. verticillioides* genome, Ppr1 and Ppr2, which are homologous to *Saccharomyces cerevisiae* Cdc55 and Rts1, respectively. In this study, we hypothesized that Ppr1 and Ppr2 are involved in the regulation of fumonisin biosynthesis and/or cell development in *F. verticillioides*, and generated a series of mutants to determine the functional role of Ppr1 and Ppr2. The *PPR1* deletion strain (Δ ppr1) resulted in drastic growth defects, but increased microconidia production. The *PPR2* deletion mutant strain (Δ ppr2) showed elevated fumonisin production, similar to the Δ cpp1 strain. Germinating Δ ppr1 conidia formed abnormally swollen cells with a central septation site, whereas Δ ppr2 showed early hyphal branching during conidia germination. A kernel rot assay showed that the mutants were slow to colonize kernels, but this is probably a result of growth defects rather than a virulence defect. Results from this study suggest that two PP2A regulatory subunits in *F. verticillioides* carry out distinct roles in the regulation of fumonisin biosynthesis and fungal development.

INTRODUCTION

The reversible phosphorylation of proteins is one of the key mechanisms for cellular regulation in both prokaryote and eukaryote cells (DeLong, 2006; Luan, 2003; Tonks, 2006). Among

the various enzymes involved in this process, phosphatases, which remove the phosphate group from its substrate, are responsible for the reversible dephosphorylation of proteins (Ceulemans and Bollen, 2004; DeLong, 2006). Protein phosphatase type 2A (PP2A) is one of the major serine/threonine (Ser/Thr) protein phosphatase families with a broad substrate specificity that plays an essential role in cellular regulatory mechanisms in eukaryotes, such as signal transduction, cell cycle regulation and development (Dickman and Yarden, 1999; Gallego and Virshup, 2005; Goldberg, 1999; Mumby and Walter, 1993). PP2A is a heterotrimer which consists of a structural A subunit, a regulatory B subunit and a catalytic C subunit. The A subunit is associated with the catalytic C subunit, forming a scaffold to which the appropriate B subunit can bind (Janssens and Goris, 2001). Significantly, we know that there are multiple isoforms of B and C subunits in eukaryotes, and studies in yeast and mammalian systems have shown that a diverse combination of PP2A heterotrimeric complexes can form at certain developmental stages or as a response to specific environmental cues (Goldberg, 1999; Virshup, 2000).

In budding yeast *Saccharomyces cerevisiae*, PP2A catalytic subunits, encoded by *PPH21* and *PPH22*, have been characterized and are known to perform redundant functions (Sneddon *et al.*, 1990). The disruption of both genes results in an extremely restricted colony on growth medium with a low survival rate. Deletion of *PPH3*, a putative PP2A catalytic subunit-like gene, in the double deletion mutant leads to lethality (Ronne *et al.*, 1991). Notably, double deletion of two PP2A catalytic subunit genes in the fission yeast *Schizosaccharomyces pombe* also results in lethality (Kinoshita *et al.*, 1996). In *Neurospora crassa*, disruption of the activity of the PP2A catalytic subunit by gene silencing or pharmacological inhibition leads to restricted hyphal growth and abnormal hyphal tip formation (Yatzkan *et al.*, 1998). Sit4 is another PP2A catalytic subunit found in yeast and other fungi that is associated with cellular development and maintenance (Arndt *et al.*, 1989; Hayashi *et al.*, 2005; Lee *et al.*, 2004). The Sit4 homologue in fission yeast, *Aspergillus nidulans* and *N. crassa* has been linked to cell shape control, mitotic cell division and actin cytoskeleton organization (Choi and Shim, 2008b; Fitzgibbon *et al.*, 2005; Shimanuki *et al.*, 1993).

There are two PP2A regulatory subunit genes in budding yeast: *CDC55*, encoding a 55-kDa regulatory B subunit (Healy *et al.*,

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1991; Zolnierowicz *et al.*, 1991), and *RTS1*, encoding the 53–74-kDa regulatory B' subunit (McCright *et al.*, 1996; Shu *et al.*, 1997). Deletion mutant strains of *cdc55* and *rts1* exhibit cold sensitivity and abnormal bud formation. One intriguing finding is that Cdc55 and Rts1 show differential localization in yeast cells, suggesting that a regulatory subunit may control specific PP2A holoenzyme functions by networking with proteins located in different cellular organelles (Gentry *et al.*, 2005). *RGB-1*, the PP2A regulatory B subunit in *N. crassa*, plays a role in hyphal growth and conidiation, which are essential for survival (Yatzkan and Yarden, 1999). The PP2A regulatory subunit is also required for normal hyphal growth in *Sclerotinia sclerotiorum*. Erental *et al.* (2007) have demonstrated that the PP2A regulatory subunit plays an important role in sclerotial development and pathogenesis. However, the functional role of PP2A constituents in filamentous fungi is not clearly understood, despite their presence in the genome and the critical roles they play in the regulation of cellular functions.

Fusarium verticillioides (Sacc.) Nirenburg (teleomorph *Gibberella moniliformis* Wineland) is a fungal pathogen of maize, known to cause maize stalk and ear rot worldwide (Desjardins, 2003; Munkvold and Desjardins, 1997). These diseases can cause economic losses in several different ways, including stalk breakage, lodging, interruption of the normal grain-filling process and premature plant death, ultimately leading to yield loss and lower grain quality. Furthermore, *F. verticillioides* produces polyketide-derived mycotoxins, namely fumonisins, in maize kernels or maize-based products. Fumonisin B₁ (FB₁), the major form of fumonisin found in nature, is known to cause various diseases when animals and humans are exposed through the consumption of contaminated corns (Marasas, 2001; Rheeder *et al.*, 1992).

Since the discovery of fumonisins, numerous efforts have been made to understand their chemistry, toxicology and biology, as well as the *F. verticillioides*–maize pathosystem. FB₁ is synthesized by a cluster of genes, designated as the *FUM* gene cluster, and such gene clusters are often involved in the synthesis of numerous mycotoxins and secondary metabolites (Proctor *et al.*, 1999, 2003; Seo *et al.*, 2001). The *FUM* gene cluster harbours over 20 genes which are co-expressed and related in fumonisin biosynthesis, including the *FUM1* gene, encoding polyketide synthase, in a 45-kb region on chromosome 1 of *F. verticillioides* (Proctor *et al.*, 2003). In addition to the *FUM* cluster, researchers have identified and characterized a number of regulatory genes to better understand the genetic mechanisms that regulate fumonisin biosynthesis in *F. verticillioides*. The majority of these genes, e.g. *FCC1*, *FCK1*, *PAC1*, *ZFR1*, *GBP1* and *GBB1*, are not linked to the *FUM* gene cluster physically (Bluhm and Woloshuk, 2006; Choi and Shim, 2008b; Flaherty and Woloshuk, 2004; Mukherjee *et al.*, 2011; Sagaram and Shim, 2007; Sagaram *et al.*, 2006a; Shim and Woloshuk, 2001), but play significant roles in fumonisin production. Notably, *FUM21*, which encodes a transcription factor that harbours a Zn(II)₂Cys₆ DNA-binding domain, is the only known

regulatory element within the *FUM* gene cluster (Brown *et al.*, 2007). However, the deletion of *FUM21* does not block fumonisin biosynthesis completely, suggesting that FB₁ biosynthesis is regulated by multiple genes and signalling pathways in *F. verticillioides*. In addition, we know that multiple environmental factors, such as limited nitrogen, acidic pH and select carbon sources (Bluhm and Woloshuk, 2005; Keller *et al.*, 1997; Shim *et al.*, 2003) influence FB₁ production. Biochemical changes inside corn kernels during the development and maturation process, specifically the production of amylopectin and dextrin (Bluhm and Woloshuk, 2005; Warfield and Gilchrist, 1999), also influence FB₁ production. These data indicate that the regulatory mechanism associated with FB₁ is complex, but we are now gaining a better understanding of the genetic mechanisms controlling fungal development and host–pathogen interactions that are interwoven with fungal secondary metabolism regulation (Bluhm and Woloshuk, 2006; Sagaram *et al.*, 2006b; Shim and Woloshuk, 2001).

In an earlier study, Choi and Shim (2008b) isolated and characterized the putative PP2A catalytic subunit gene in *F. verticillioides*, designated *CPP1*, encoding a protein highly similar to yeast Sit4. The gene deletion mutant (Δ cyp1) showed elevated levels of FB₁ production and the derepression (or up-regulation) of *FUM1* gene expression. The mutant also showed severe developmental defects, e.g. hyphal swelling and reduced colony growth. The pleiotropic phenotypes observed in Δ cyp1 suggested that the PP2A catalytic subunit in *F. verticillioides* is associated with multiple cellular functions, and subsequently raised the question of how the PP2A catalytic subunit regulates substrate specificity and ultimately influences diverse cellular functions. In this study, we hypothesized that PP2A regulatory subunits play a critical and, perhaps, distinct role in determining the function of the PP2A complex in *F. verticillioides*. We identified two genes encoding putative PP2A regulatory subunits in the *F. verticillioides* genome, and designated these as *PPR1* and *PPR2* (putative Protein Phosphatase Regulatory subunit 1 and 2). We generated mutations in the two genes, and investigated the impact of each mutation in *F. verticillioides*. The deletion of *PPR1* and *PPR2* resulted in distinct phenotypes associated with conidiation, FB₁ production and cell morphology, suggesting that Ppr1 and Ppr2 selectively regulate different subsets of signalling pathways in the fungal pathogen.

RESULTS

PP2A regulatory B subunits in *F. verticillioides*

To identify the protein phosphatase regulatory subunits in *F. verticillioides*, we initiated an *in silico* analysis with two published PP2A regulatory subunits from *S. cerevisiae*: Cdc55 (GenBank accession no. NM_001181055) and Rts1 (GenBank accession no. NM_001183433). A study of the *F. verticillioides* genome (Ma *et al.*, 2010) revealed the presence of two putative

PP2A regulatory subunits. *PPR1* (FVEG_01508) is predicted to encode a 474-amino-acid protein homologous to Cdc55, and is located in supercontig 1 on chromosome 1, specifically from sequence 4 599 671 to 4 662 020. *PPR2* (FVEG_04543) is predicted to encode a putative 658-amino-acid Rts1 homologue, and is found in supercontig 5, specifically from sequence 665 726 to 667 909. CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and Pfam (pfam.sanger.ac.uk) searches showed that Ppr1 and Ppr2 harbour a WD40 domain and B56 domain, also found in Cdc55 and Rts1, respectively. Protein sequence identity between Cdc55 and Ppr1 was 65%, and the conserved B56 domain identity between Rts1 and Ppr2 was 63% when using the WU-BLAST algorithm (<http://www.yeastgenome.org>) (Fig. S1, see Supporting Information).

Deletion of *PPR1* and *PPR2* in *F. verticillioides*

To determine the functional roles of PP2A regulatory subunits in *F. verticillioides*, we generated deletion mutants. The *PPR1* split-marker gene disruption construct, which contains 5' and

3' flanking regions of the *PPR1* gene with the hygromycin resistance gene as a selectable marker (*HPH*, *hygromycin B phosphotransferase*), was transformed into wild-type *F. verticillioides* 7600 protoplasts (Fig. 1A). Three independent hygromycin-resistant knockout mutants were isolated and confirmed by polymerase chain reaction (PCR) (data not shown) and Southern analysis (Fig. S2A, see Supporting Information). Genomic DNA samples were digested with *EcoRV*, and the result showed a 3.2-kb band in select transformants, indicating that the *PPR1* locus had been replaced with the *HPH* marker. The wild-type, as expected, displayed a 0.6-kb band. In addition, *PPR1* gene deletion was confirmed by Northern analysis, in which transcriptional expression was not detectable (Fig. 2A). After a series of phenotype evaluations, e.g. growth rate, culture morphology and secondary metabolite production (Choi and Shim, 2008b; Mukherjee *et al.*, 2011; Sagaram *et al.*, 2007), we selected strain b46-5 (herein designated Δ ppr1) from a pool of *PPR1* gene deletion strains for further molecular characterization.

PPR2 deletion was first generated in strain SF41, taking advantage of the increased homologous recombination efficiency (Choi

Fig. 1 Homologous recombination strategy to generate Δ ppr1, Δ ppr2 and Δ ppr1/ Δ ppr2 gene deletion mutations in *Fusarium verticillioides*. (A) *PPR1* gene deletion was performed in the wild-type 7600 strain using the split-marker strategy with *hygromycin B phosphotransferase* (*HPH*) as the selectable marker. The *HPH* gene was partially amplified (*HP* and *PH*) and joined to the 5' left flanking (*LF*) DNA and 3' right flanking (*RF*) DNA, respectively. (B) *PPR2* gene deletion was performed in the SF41 (Δ Ku70) strain with *HPH* as the selectable marker. (C) *PPR2* gene deletion (re-knockout) was performed in the wild-type 7600 strain with the *geneticin-resistance gene* (*GEN*) as the selectable marker. (D) The Δ ppr1/ Δ ppr2 double mutant was created by performing *PPR1* gene deletion in the Δ ppr2 strain using the split-marker strategy with *HPH* as the selectable marker. The dotted line underneath the *PPR1* or *PPR2* gene represents the genomic region used as a probe for Southern and Northern analyses. E, *EcoRV* restriction site (A, D); N, *NcoI* restriction site (B, C).

Fig. 2 Northern analyses of *PPR1*, *PPR2* and *CPP1* expression in *Fusarium verticillioides* wild-type (WT), Δ ppr1, Δ ppr2 and Δ cpp1 strains. Total RNA samples (12 μ g) were subjected to electrophoresis in 1.2% denaturing gel, and 32 P-labelled DNA fragments of *PPR1* (A), *PPR2* (B) and *CPP1* (C) were used to determine gene expression levels. The gels were stained with ethidium bromide to confirm the uniformity of loading (rRNA).

and Shim, 2008a) (Fig. 1B). Six independent *PPR2* deletion mutants, which were hygromycin resistant, were confirmed by Southern analysis with the 5' flanking region fragment of *PPR2* as a probe, and these mutant strains shared indistinguishable phenotypes on V8 agar (data not shown). As anticipated, Southern analysis with *NcoI*-digested genomic DNA samples resulted in a 3.3-kb band in the mutants, whereas the wild-type strain displayed a 11.6-kb band (Fig. S2C). Northern analysis confirmed that the expression of *PPR2* was abolished in the mutant (Fig. 2B). Among the deletion strains, p4 (herein designated Δ ppr2) was selected for further characterization (Fig. S2A).

To further validate that the mutant phenotypes observed in Δ ppr1 and Δ ppr2 are a result of the single gene deletion of *PPR1* and *PPR2*, respectively, we once again knocked out each gene in the wild-type strain 7600 using a different selectable marker. This was because of the inexplicable difficulty in generating protoplasts from Δ ppr1 to perform complementation of these mutants through transformation. For these experiments, we used the homologous recombination strategy employed for *PPR2* gene deletion (Fig. 1C), but with the *geneticin-resistance gene* (*GEN*) as the selectable marker. Deletion of the *PPR1* gene was confirmed by Southern analysis (Fig. S2B). This mutant, designated rp160, showed indistinguishable phenotypes from the b46-5 strain (Δ ppr1) when tested on select media (Fig. S3B,C, see Supporting Information), suggesting that the deletion of the *PPR1* gene is responsible for the aberrant phenotypes observed in earlier Δ ppr1 strains. Similarly, three *PPR2* deletion mutants generated with *GEN* selection, designated pg18, pg103 and pg108, were confirmed by Southern analysis (Fig. S3D), and exhibited indistinguishable phenotypes on multiple media when compared with the p4 (Δ ppr2) strain (Fig. S3). These results indicate that the *PPR2* deletion is directly responsible for the altered phenotypes observed in the Δ ppr2 mutants.

Subsequently, we selected the pg18 strain (*PPR2* re-knockout strain in the wild-type background with *GEN* as the marker) and employed the split-marker constructs used to delete *PPR1*, creating Δ ppr1 Δ ppr2 double mutants (Fig. 1D). We isolated multiple strains showing both hygromycin and geneticin resistance. Two strains, bp106 and bp166, were confirmed as *PPR1* deletion mutants by Southern analysis (Fig. S2E).

Northern blots revealed that the expression of *PPR1* in Δ ppr2 and *PPR2* in Δ ppr1 was similar to the expression observed in the wild-type (Fig. 2A,B). Intriguingly, we found that *CPP1* expression in Δ ppr2 was elevated when compared with that in Δ ppr1 and the wild-type (Fig. 2B). Similarly, *PPR2* showed a higher level of expression in Δ cpp1 than in the wild-type (Fig. 2C). However, further studies are needed to investigate whether *PPR2* and *CPP1* regulate each other in a reciprocal manner, or perhaps compensate for the loss of one another in the mutants.

Phenotypes of deletion strains of PP2A regulatory subunits in *F. verticillioides*

Deletion mutations resulted in a growth deficiency in *F. verticillioides* on V8 agar, potato dextrose agar (PDA) and defined agar medium (Fig. 3A,B). The Δ cpp1 mutant was also inoculated alongside to compare the role of the PP2A regulatory B subunits and the catalytic C subunit on radial growth in *F. verticillioides*. Although Δ cpp1 exhibited a reduced growth when compared with the wild-type, the growth defect in Δ cpp1 was not as dramatic as that observed in Δ ppr1. The growth of Δ ppr1 was drastically reduced, and was approximately 10%–14% of that of the wild-type (Fig. 3B). The growth defect was not as severe in Δ ppr2, but was still approximately 50% of the wild-type on all solid media tested (Fig. 3B). Significantly, both Δ ppr1 and Δ ppr2 showed no significant difference in mycelial mass (dry weight) in liquid medium

Fig. 3 Colony morphology and growth of the wild-type (WT) and mutant strains on various solid media: potato dextrose agar (PDA), V8 agar and defined medium (DM). (A) Strains were point inoculated with an agar block (0.5 cm in diameter) and incubated for 7 days at 25 °C. (B) Relative growth (%) of mutants compared with that of the wild-type strain. Results are means $\pm$ standard error of four biological replicates.

(Fig. 4). In addition, the $\Delta ppr1$ strain secreted an unknown red-to-crimson pigment into agar medium during growth (Fig. S3).

To test whether PP2A regulatory subunits in *F. verticillioides* affect asexual reproduction, we monitored conidia production (Fig. S4A, see Supporting Information). As a result of the difference in vegetative growth observed in the two mutants, we opted to measure the number of conidia per designated radial growth

Fig. 4 Growth and morphology of wild-type (WT) and mutant strains on yeast extract–peptone–dextrose (YEPD) agar and in YEPD broth. (A) Growth and colony morphology on YEPD. (B) Spores (10^6) of fungal strains were inoculated and cultured in YEPD broth for 7 days, and subsequently filter harvested. Fresh weight results are the means $\pm$ standard deviation of three biological replications. (C) Microscopic observation ($200\times$ magnification) of wild-type, $\Delta ppr1$, $\Delta ppr2$ and $\Delta cpp1$ strains after 7-day shaking incubation in YEPD broth. Strain $\Delta ppr1$ showed more condensed and extensively branched mycelia when cultured in YEPD broth.

Fig. 5 (A) Microconidia production per unit area in wild-type (WT), $\Delta ppr1$, $\Delta ppr2$ and $\Delta cpp1$ strains. Strains were point inoculated onto KCl agar plates with an agar block (0.5 cm in diameter), and incubated for 7 days at 25 °C. Conidia were harvested and quantified with a haemocytometer. The area of radial growth was measured and converted to square millimetres. As the standard deviations of the area of all wild-type and mutant strains could be ignored, the number of conidia were divided by the area (mm^2) of radial growth. Four biological replications were performed to obtain standard deviations. (B) Relative fumonisin B₁ (FB₁) production of wild-type and mutant strains was calculated as the mean FB₁ production (ppm) from each strain divided by the percentage ergosterol production of each mutant strain relative to the ergosterol production of the wild-type strain.

area on agar plates (Fig. 5A). Surprisingly, $\Delta ppr1$ produced approximately four times more conidia per unit area than the wild-type, which was in contrast with its severe growth defect on solid media. $\Delta ppr2$ produced significantly fewer conidia, approximately one-tenth of the wild-type. We also determined that there was no statistically significant difference in the number of conidia between $\Delta cpp1$ and the wild-type. These data suggest that PP2A regulatory subunits Ppr1 and Ppr2 play negative and positive roles, respectively, in asexual development, independent of the PP2A catalytic subunit in *F. verticillioides*.

We also tested sexual reproduction in the mutant strains. *Fusarium verticillioides* 7598 was used as the opposite mating type of the wild-type progenitor (strain 7600) and the deletion mutants in sexual crosses (Sagaram *et al.*, 2007). All crosses were successful and resulted in perithecia formation and viable ascospores, suggesting that PP2A subunits are not required for sexual reproduction in *F. verticillioides* (data not shown).

Role of PPR1 and PPR2 in FB₁ production and kernel rot

As shown in our previous study (Choi and Shim, 2008b), $\Delta cpp1$ produces about 10 times more FB₁ than the wild-type. In this study, high-performance liquid chromatography (HPLC) analyses showed that $\Delta ppr2$ produces a higher level of FB₁ compared with that of the wild-type, whereas $\Delta ppr1$ produces significantly less FB₁ than the wild-type (Fig. S4B). Subsequently, we assayed ergosterol levels to determine the amount of growth of each strain in corn kernels (Fig. S4C). This was performed to standardize FB₁ production to fungal mass in infected corn kernels. The mean FB₁ level of each strain was divided by the mean of the ergosterol content from each strain. We determined that the FB₁/ergosterol ratio in $\Delta ppr2$ was 10 times greater than that of the wild-type strain, whereas $\Delta ppr1$ produced approximately 50% less FB₁

when compared with the wild-type strain (Fig. 5B). These results suggest that *PPR1* and *PPR2* regulate FB_1 production in *F. verticillioides* in a positive and negative manner, respectively.

After testing for FB_1 production and fungal colonization in autoclaved corn kernels, we also investigated whether *PPR1* and *PPR2* were important for kernel rot virulence in *F. verticillioides*. We inoculated wild-type, $\Delta ppr1$ and $\Delta ppr2$ strains on surface-sterilized B73 corn kernels, and observed the level of colonization after 7 days of incubation. All strains were able to colonize B73 kernels (Fig. S5A, see Supporting Information) and, although we saw a difference in the level of fungal growth on kernels, subsequent ergosterol analyses showed that this difference was probably a result of growth deficiency in the mutant strains (Fig. S5B).

Impact of PP2A regulatory subunits on conidia germination

We frequently observed abnormal conidia germination phenotypes in the PP2A regulatory subunit mutants (Fig. 6A). Approximately 20% of $\Delta ppr1$ conidia grew isotropically to form an aberrantly swollen conidium, although germlings maintained polarity in these cells. The swollen conidium phenotype was more severe in $\Delta ppr1$ than in $\Delta cpp1$ (Choi and Shim, 2008b). Typically, a *F. verticillioides* single-celled and oval-shaped microconidium germinates by first forming one germ tube, with the secondary germ tubes typically emerging from the opposite 180° from the primary germ tube. In addition, the double mutant showed both swelling and a secondary germ-tube emergence pattern observed

in $\Delta ppr1$ and $\Delta ppr2$, respectively. In the $\Delta ppr2$ strain, approximately 20% of conidia sent the second germ tube from a site adjacent to the primary germ tube (Fig. 6B). These results suggest that *Ppr1* is important for the maintenance of growth exclusively at the germling tip, and that *Ppr2* plays a key role in site selection of the germ tube in *F. verticillioides*.

Localization of *Ppr1* and *Ppr2* in *F. verticillioides*

To visualize the localization of *Ppr1* *in vivo*, a *Ppr1::sGFP* construct (*sGFP*, synthetic green fluorescent protein) was introduced into the wild-type strain because of the difficulty in generating $\Delta ppr1$ protoplasts. We isolated two transformants in which the native *PPR1* locus was replaced with the *Ppr1::sGFP* construct, which was confirmed by PCR and Southern analysis (data not shown). In these strains, we observed a low level of GFP signal throughout the cytoplasm (Fig. S6, see Supporting Information). For *Ppr2*, we were able to generate protoplasts from $\Delta ppr2$ strain pg18, and introduced the *Ppr2::sGFP* construct into mutant protoplasts. We isolated a total of 16 transformants showing the restored, wild-type phenotype; however, we were unable to see or image GFP expression in these strains (data not shown). As an alternative approach, we generated a constitutively expressed construct *gpdA(p)::PPR2::sGFP*, which was under the control of the *A. nidulans gpdA* promoter, and transformed it into pg18 protoplasts. We isolated 15 transformants showing a wild-type (rescued) phenotype, GFP expression and the presence of *PPR2* by PCR screening (data not shown). Even with the constitutive promoter, a low level

Fig. 6 (A) Germinating conidia of wild-type (WT), $\Delta cpp1$, $\Delta ppr1$, $\Delta ppr2$ and $\Delta ppr1\Delta ppr2$ strains in yeast extract–peptone–dextrose (YEPD) liquid medium. Each strain was incubated for 15 h at 25 °C before the observation. Scale bar, 10 μ m. (B) Time-lapsed observation of $\Delta ppr2$ conidium germination. The first panel demonstrates the first detectable emergence of the germ tube, and each subsequent panel represents exactly 20 min after the panel to its left. Scale bar, 10 μ m.

of GFP was noted throughout the cytoplasm and localization was enriched at the septa (Fig. S6).

DISCUSSION

Holoenzyme formation of PP2A subunits is necessary for the multifunctional PP2A complex to perform precise and specific activities in certain developmental stages in eukaryotes (Goldberg, 1999; Mayer-Jaekel and Hemmings, 1994; Virshup, 2000). Therefore, the identification of the role of each subunit can provide a fundamental understanding of how PP2A regulates important cellular functions. Two PP2A regulatory subunits, B and B', have been characterized in *S. cerevisiae*: Cdc55p and Rts1p, respectively. In filamentous fungi, a select number of B-type regulatory subunits have been characterized, e.g. in *N. crassa* and *S. sclerotiorum* (Erental *et al.*, 2007; Yatzkan and Yarden, 1999). Recently, the B' regulatory subunit and the catalytic subunit in *F. graminearum* have been characterized, and the study showed that the PP2A complex plays an important role in vegetative growth, sexual and asexual development, mycotoxin biosynthesis and pathogenicity (Kim and Yun, 2012). In this study, we identified and characterized both PP2A regulatory subunit genes, *PPR1* and *PPR2*, with the goal of elucidating the role of the PP2A complex in the regulation of the cellular functions of *F. verticillioides*. The generation of a *PPR2* deletion mutant in *F. verticillioides* is noteworthy because the characterization of the regulatory B subunit, but not the B' subunit, has been reported in filamentous fungi.

Deletions of PP2A subunits resulted in diverse phenotypes, supporting the idea that each PP2A subunit has a different role. Among these subunits, it is plausible that *PPR1*, which encodes the PP2A regulatory B subunit, plays a major role in hyphal growth in *F. verticillioides*, as deduced from the severe radial growth defects observed in the Δ ppr1 strain. A similar outcome was observed when *RGB-1* was mutated in *N. crassa* (Yatzkan and Yarden, 1999). However, in addition to the defective hyphal growth on solid media, we observed Δ ppr1 cell compartments that were shorter and wider in diameter. Yellman and Burke (2006) showed that Cdc55, a homologue of Ppr1, was required for proper spindle checkpoint during mitosis in *S. cerevisiae*. However, our preliminary 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33257 staining of the mutants did not reveal visual differences in morphology or distribution of nuclei compared with the wild-type (data not shown). Interestingly, there was no statistically significant difference in the biomass of Δ ppr1 and the wild-type strain when cultured in yeast extract–peptone–dextrose (YEPD) broth. This was unexpected when considering the growth of Δ ppr1 on solid media (Figs 3 and 4). However, we were able to observe a number of compact hyphal clusters in Δ ppr1 liquid culture (Fig. 4C), and it is reasonable to suggest that the Δ ppr1 growth defect observed in solid agar does not hamper fungal mass production in shaking, liquid culture.

In contrast with Δ ppr1 growth, Δ cyp1 showed a less pronounced growth defect in all media tested. This outcome demonstrates that a PP2A regulatory subunit is more critical for PP2A complex function in filamentous fungi. In contrast with mammalian systems, there is a lack of diversity in PP2A regulatory subunits. For catalytic subunits, it has been shown that fungi harbour a subset of phosphatase catalytic subunits that can compensate for the loss of another (Kosmidou *et al.*, 2001; Sneddon *et al.*, 1990). For instance, triple deletion of *PPH* genes in budding yeast was initially determined as lethal, but the fact that the triple deletion mutant was viable in high-glucose conditions suggests that an alternative, yet to be identified, protein can rescue the triple mutation (Hu and Ronne, 1994). In addition, this study showed that an over-expression of the *PAM1* gene, the PP2A multicopy suppressor, increased the survival rate of the *PPH* triple deletion mutant at lower glucose concentration. It is also reasonable to postulate that there are alternative PP2A catalytic subunits that can substitute for Cyp1 and maintain a certain level of PP2A complex function.

Mild growth defects observed in the Δ ppr2 mutant also imply that the regulatory B' subunit is also involved in hyphal growth in *F. verticillioides*. In yeast, slower and reduced growth in the B' subunit mutant strain was repeatedly observed, which led to the suggestion that the PP2A regulatory B' subunit is associated with stress responses, regulation of cell cycle progression, septum positioning and cell size control (Shu *et al.*, 1997; Tanabe *et al.*, 2001). One other interesting aspect of the B' subunit is that it can suppress the activity and substrate specificity of PP2A AC dimer (Usui *et al.*, 1988). Therefore, the severe defective growth in Δ ppr1 could be a result of the loss of balance between B and B' regulatory subunits. In fission yeast, the B subunit over-expression strain exhibited phenotypes that partially overlapped with the B' deletion strain, and the B' over-expression strain shared the abnormalities shown in the B deletion strain (Kinoshita *et al.*, 1996; Tanabe *et al.*, 2001). However, the inhibitory activity of the PP2A regulatory B' subunit was not demonstrated in filamentous fungi. The investigation of how, or whether, *PPR1* and *PPR2* over-expression influences the cell compartment may provide a better understanding of how PP2A functions are controlled in *F. verticillioides* and other filamentous fungi.

Our results suggest that Ppr2 is the regulatory subunit that influences the PP2A complex to regulate FB₁ synthesis in *F. verticillioides*. In budding yeast, deletion of *RTS1* resulted in the constitutive expression of amino acid permeases (AAPs), which are normally activated when the uptake of amino acids is needed (Eckert-Boulet *et al.*, 2006). As FB₁ production in *F. verticillioides* is facilitated under nitrogen stress (Shim and Woloshuk, 1999), we can hypothesize that the AB'C form of the PP2A complex is involved in the recognition of a nitrogen stress signal in *F. verticillioides*, whereas the ABC form functions in an opposite manner. Furthermore, Δ ppr1 produced heavy pigmentation, suggesting that PP2A negatively regulates a majority of secondary

metabolites (Figs 3 and S3). Secondary metabolites are often produced by a cluster of genes, e.g. the *FUM* gene cluster, and are regulated by transcription factors (Yu and Keller, 2005). We can postulate that Ppr1-driven PP2A activity is located upstream of these transcription factors to prevent the unnecessary production of secondary metabolites. However, as we have yet to characterize the specific secondary metabolites overproduced in Δ ppr1, it is difficult to speculate further on how PP2A is involved in the regulation of various secondary metabolism processes in *F. verticillioides*.

Although macroconidia production was one of the noticeable phenotypes in Δ cpp1 (Choi and Shim, 2008b), suggesting that the PP2A catalytic subunit is involved in the regulation of macroconidia–microconidia equilibrium, macroconidia were not observed in the Δ ppr1 or Δ ppr2 strains. Involvement of the PP2A regulatory B subunit in macroconidia production has been observed previously in the *rgb-1* mutant in *N. crassa* (Yatzkan and Yarden, 1999). However, our results suggest that Cpp1, not the regulatory subunits, is the key player in the macroconidia–microconidia equilibrium in *F. verticillioides*. However, we observed distinct microconidia morphology and germination patterns in the three mutant strains (Fig. 6A). Overproduction of microconidia in Δ ppr1 resembles the increased arthroconidia production in the *rgb-1* mutant. *Rgb-1* is required for major constriction formation in *N. crassa* macroconidia (Yatzkan and Yarden, 1999). Microconidia overproduction was also observed when *FvVE1*, the orthologue of *A. nidulans* *veA*, was deleted in *F. verticillioides* (Li *et al.*, 2006), which is similar to one of the Δ cpp1 phenotypes. Therefore, we can hypothesize that Cpp1 and Ppr1 are located downstream of *FvVE1*, but perform different regulatory functions. However, the genetic link between *FvVE1* and the PP2A complex has not been determined to date (Choi and Shim, 2008b).

Approximately 20% of germinating microconidia in Δ ppr1 exhibited abnormal morphology, notably central septation (Fig. 6A). Septation is a product of cell division in filamentous fungi. In fission yeast, during mitotic cell division, Plo1 kinase accumulates and leads to increased kinase activity (Tanaka *et al.*, 2001). Considering the important role of the PP2A regulatory B subunit in mitosis, we can hypothesize that Ppr1 is involved in the regulation of Plo1-like kinase activity. However, it is unclear whether the ABC heterotrimer of PP2A controls directly the factors inducing septation. Septation in germination of the Δ ppr2 strain was normal, and this result contrasts with the multi-septation phenotype of the B' subunit deletion mutant in fission yeast (Tanabe *et al.*, 2001). Interestingly, the conidia of Δ ppr2 developed a second germ tube just after germination. This is similar to the lateral branching pattern described by Harris (2008), and is generally associated with septation. In Δ ppr2, early branching was only observed at the tip at which the primary germ tube emerged, suggesting an orientation change of the axis of polarity. Further

observations are required to investigate the detailed role of Ppr2 in branching during early germination in *F. verticillioides*.

Determination of the localization of the two PP2A regulatory subunits could be an important step in understanding the intricate mechanisms of PP2A involvement in various cellular functions. Although the complete PP2A complex is essential for substrate specificity and cellular function, the localization of each regulatory subunit will help us to understand how and where PP2A functions are performed. The dynamic localization pattern of each regulatory subunit has been monitored in *S. cerevisiae* (Gentry and Hallberg, 2002), and Cdc55p and Rts1p localized to different sites during mitosis. In our study, we observed different localization patterns for Ppr1 and Ppr2, suggesting that these regulatory subunits dictate the spatial disposition of the PP2A complex (Fig. S6). Notably, recent published reports have led us to hypothesize that *F. verticillioides* and other filamentous fungi harbour a third putative PP2A regulatory subunit that shares a high similarity to the striatin family of proteins (Bloemendal *et al.*, 2012; Shim *et al.*, 2006; Simonin *et al.*, 2010; Wang *et al.*, 2010). Significantly, a study by Wang *et al.* (2010) showed that this protein is primarily localized to the endomembrane in *A. nidulans*, particularly the endoplasmic reticulum. These data led us to hypothesize that the spatial distribution of the PP2A complex in *F. verticillioides*, as well as other filamentous fungi, is dictated by its regulatory subunit, and thus interacts with a unique subset of substrates for distinct cellular function (Fig. 7). Further characterization of the interacting proteins and substrates of the PP2A complex will help us to better understand the role of PP2A in *F. verticillioides*.

EXPERIMENTAL PROCEDURES

Fungal strains and culture media

Fusarium verticillioides strain 7600 (also known as M3125, genotype MAT1-1) (Fungal Genetics Stock Center, Kansas City, KS, USA) was used as the wild-type strain in this experiment. We also used SF41, a *FvKU70* gene deletion strain, as an alternative to the wild-type strain when conducting gene deletion experiments (Choi and Shim, 2008a). All fungal strains used in this study were stored in 30% glycerol at -80°C . For inocula and routine maintenance, conidia were harvested from the fungus grown on V8 juice agar (200 mL of V8 juice, 3 g of CaCO_3 and 20 g of agar powder per litre) at 25°C for 7 days. For genomic DNA extraction, strains were grown in 100 mL of YEPD medium (3 g of yeast extract, 10 g of peptone and 20 g of dextrose per litre) in 250-mL glass flasks on a rotary shaker at 125 rpm for 3 days. For total RNA extraction, mycelia grown in YEPD medium for 3 days were harvested using Whatman no.1 filter paper (Whatman Inc., Clifton, NJ, USA), and were subsequently inoculated into 100 mL of defined medium (DM; 1 g of $\text{NH}_4\text{H}_2\text{PO}_4$, 3 g of KH_2PO_4 , 2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g of NaCl and 40 g of sucrose per litre), which is known to support FB₁ production (Choi and Shim, 2008b; Shim and Woloshuk, 2001). Colony morphology was visually compared on PDA (BD Diagnostic Systems, Sparks, MD, USA), V8 agar and DM agar. The radius of expanding

Fig. 7 Proposed spatial distribution of protein phosphatase type 2A (PP2A) regulatory subunits in *Fusarium verticillioides*. Based on the microscopic observation of Ppr1::GFP and Ppr2::GFP localization in this study, we hypothesize that Ppr1 is distributed throughout the cytoplasm, whereas Ppr2 is concentrated near the septa. The third putative PP2A regulatory subunit in filamentous fungi, Fsr1/StrA/Pro11, is suggested to be localized near the endoplasmic reticulum and the nuclear envelope.

mycelia on plates was measured for 7 days with three replications for each strain. For microconidia counts, agar blocks of fungal strains from a V8 agar culture were transferred to KCl agar plates (6 g of KCl and 15 g of agar powder per litre). After 7 days of incubation, the conidia were harvested with 0.1% Triton X-100 and counted using a haemocytometer.

Nucleic acid manipulation and PCR

Genomic DNA was extracted using the OmniPrep genomic DNA extraction kit (G Biosciences, Maryland Heights, MO, USA). Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's suggested protocol. Genomic DNA (10 µg) was digested with appropriate restriction endonucleases for Southern hybridization. Gel electrophoresis, gel blotting and hybridization for Southern and Northern analyses were performed following standard procedures (Choi and Shim, 2008b). The probes used in Southern and Northern hybridization experiments were ³²P-labelled with a Prime-It Random Primer Labelling Kit (Stratagene, La Jolla, CA, USA).

PCR amplification of DNA was performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, CT, USA). PCR amplification was performed in a total volume of 50 µL with Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) or Expand Long Polymerase (Roche, Indianapolis, IN, USA). The usual PCR conditions included 2 min of pre-denaturation at 94 °C, followed by 30 cycles of

30 s of denaturation at 94 °C, 30 s of annealing at 58 °C and 1–2 min of extension at 72 °C for Taq DNA polymerase (68 °C for Expand Long Polymerase). After 30 cycles, 10 min at 72 °C was carried out for final extension. Single- or double-joint PCR was performed using Expand Long Polymerase following the manufacturer's suggested protocol. PCR products were purified using a Wizard SV gel and PCR purification system (Promega, Madison, WI, USA). The primers used in this study are listed in Table S1 (see Supporting Information).

Construction of transforming vectors

We generated *PPR1* and *PPR2* gene deletion constructs with a split-marker approach and a standard homologous recombination approach, respectively (Sagaram *et al.*, 2007; Yu *et al.*, 2004). For both deletion constructs, DNA fragments of the 5' and 3' flanking regions of each gene were amplified from genomic DNA of the M3125 strain using the primers listed in Table S1. For the *PPR1* deletion construct, we amplified partial, but overlapping, *HPH* genes, named *HP* and *PH* fragments, which have about 300-bp overlapped sequences, using the primer pairs HYG-F/HY-R and YG-F/HYG-R, respectively (Fig. 1A). PCR fragments of the 5' and 3' flanking region of the *PPR1* gene were fused with *PH* and *HP* fragments, respectively, using appropriate primers. For the *PPR2* deletion or re-knockout constructs, *HPH* and *GEN* genes were amplified from pBP15 and pBS-G, respectively (Fig. 1), using the primer pair M13F and M13R. The marker (*HPH* or *GEN*) fragments were flanked by the 5' and 3' flanking region of the *PPR2* gene, and were subsequently amplified using nested primers. All DNA fragments amplified by PCR were transformed directly into the fungal protoplast. To create the double deletion mutant (Δ ppr1 Δ ppr2), we used split-marker constructs generated earlier for *PPR1* gene deletion (with *HPH* as the selectable marker) in a Δ ppr2 strain (Δ ppr2::*GEN*) (Fig. 1D).

For the *in vivo* localization of Ppr1 and Ppr2, we constructed Ppr1::sGFP, Ppr2::sGFP and *gpdA*(p)::Ppr2::sGFP, which were driven by their native or *A. nidulans GpdA* promoter. For the construct under the control of the native promoter, sGFP, with five glycine–alanine repeat (GA-5) sequences attached at the N-terminus as a linker, was amplified from gGFP using the primers sGFP-F-link and sGFP-R. For Ppr1::sGFP, the 940-bp fragment of the open reading frame, including the GA-5 linker of *PPR1*, and the 936-bp fragment downstream of *PPR1* were amplified using the primer pairs PPR1-SEG-F/PPR1-SEG-R2 and PPR1-3'-GFP-F/PPR1-3'-R, respectively. Using the double-joint PCR method, Ppr1::sGFP carrying sGFP in the C-terminus of *PPR1* was amplified using the nested primers PPR1-NES-F and PPR1-NES-R. For Ppr2::sGFP, 3485-bp *PPR2* fragments, which include the native promoter and the open reading frame without the stop codon, were amplified using the primers PPR2-Prom-F and PPR2-SEG-R with the complete GA-5 linker in the C-terminus. The *PPR2* PCR fragment and the sGFP fragment were fused using a single-joint PCR method with primers PPR2-Prom-N-F and sGFP-R. To generate the *gpdA*(p)::Ppr2::sGFP strain, the *gpdA* promoter was amplified using primers *gpdA*-F-Hyg and *gpdA*-R from sGFP plasmid DNA (Maor *et al.*, 1998). The 2189-bp *PPR2* gene without the stop codon was amplified using primers PPR2-F-GPDA and PPR2-SEG-R with the GA-5 linker. *gpdA*(p)::Ppr2::sGFP was fused by a double-joint PCR strategy. Three PCR products were co-transformed with pBP15 for selection on hygromycin-amended medium.

Fungal transformation

Fusarium verticillioides protoplasts were generated following the protocol described by Shim & Woloshuk (2001) with modifications, namely the replacement of mureinase with driselase (Sigma-Aldrich, St. Louis, MO, USA). For the preparation of protoplast, 10^8 spores of M3125, SF41 or select mutant strains were grown in YEPD liquid medium for 14–18 h at 25 °C with agitation. Transformed protoplasts were selected on regeneration medium containing either 100 µg/mL of hygromycin B (Calbiochem, La Jolla, CA, USA) or 150 µg/mL of geneticin, G418 sulphate (Cellgro, Manassas, VA, USA), depending on the marker used in the experiment. Antibiotic-resistant colonies that grew on the regeneration medium were selected and further screened.

FB₁, ergosterol and kernel rot analyses

FB₁ analysis was conducted as described previously (Shim and Woloshuk, 1999) with modifications. Fungal strains were grown on cracked corn medium (B73 genotype; 2 g dry weight) in a 20-mL glass vial (VWR, West Chester, PA, USA) for 14 days at room temperature. For FB₁ extraction, 10 mL of acetonitrile–water (1:1, v/v) were added to each vial and incubated at room temperature on a laboratory bench overnight; 2 mL of crude extract was purified using equilibrated Prep Sep SPE C18 columns (Fisher Scientific, Pittsburgh, PA, USA). The FB₁ concentrations of the samples were analysed using a Shimadzu LC-20AT HPLC system (Shimadzu Scientific Instruments Inc., Kyoto, Japan) equipped with a 4.6 mm × 150 mm analytical Zorbax ODS column (Agilent Technologies, Santa Clara, CA, USA) and a Shimadzu fluorescence detector. The detection wavelength was 335 nm for excitation and 440 nm for emission. The HPLC system was operated following the protocol described previously (Shim and Woloshuk, 1999).

To measure fungal growth in corn kernels, we analysed ergosterol. The extraction procedure was conducted following Kim and Woloshuk (2008). After a 14-day incubation of fungal strains in cracked corn medium, 10 mL of chloroform–methanol (2:1, v/v) were added to each vial and incubated at 25 °C for 24 h without agitation. The supernatant was collected after centrifugation, filtered through a 0.45-µm nylon membrane (Pall Life Science, Ann Arbor, MI, USA), and injected directly into the HPLC system equipped with a 4.6U ODS column (250 mm × 4.6 mm; Agilent Technologies). Ergosterol was detected with a Shimadzu SPD-20A UV–visible detector at a wavelength of 282 nm. The mean ergosterol production was generated from four replications, and an analysis of variance (ANOVA) between groups test confirmed that each mean value was statistically significantly different ($P < 0.001$).

Corn seeds (genotype B73) were used to determine the virulence of wild-type, Δ ppr1 and Δ ppr2 strains, as described previously (Mukherjee *et al.*, 2011; Sagaram *et al.*, 2007). Briefly, maize kernels were surface sterilized with bleach (containing 6% sodium hypochlorite) for 15 min, and rinsed with sterilized, distilled water at least five times. In order to provide an infection site for fungal inoculation, embryos of each kernel were cut longitudinally (0.5 cm) using a razor blade to a depth of about 0.5 mm. Seeds were dried with paper towels and placed in a sterile aluminium foil cup (diameter, 3.5 cm), which was placed in a glass Petri dish with water-soaked cheesecloth and a filter paper (Whatman, Maidstone, Kent, UK) to retain humidity. Fungal colonization of kernels was

observed after a 7-day incubation at 25 °C with a 12-h light/12-h dark cycle and, subsequently, ergosterol analysis of infected seeds was performed. All experiments were performed with at least four independent seeds per fungal strain.

Microscopy

Microscopic imaging of fungal development was performed on an Olympus BX51 microscope (Olympus America, Melville, NY, USA). A detailed description of the features used for imaging from this microscope has been published previously (Choi and Shim, 2008b; Upadhyay and Shaw, 2006). For images of hyphal growth phenotypes, spores of each strain were grown in YEPD for 15 h at room temperature. For GFP visualization, spores, collected from V8 agar medium, were incubated on coverslips with defined medium. After 13 h, living cells expressing GFP were observed under the microscope with an appropriate filter set. An Olympus SZX12 stereomicroscope system equipped with a digital camera was used to observe the fungal colonization of corn kernels.

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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 (A) Amino acid alignment of *Saccharomyces cerevisiae* Cdc55 and *Fusarium verticillioides* Ppr1. Identical amino acids and similar amino acids are highlighted with dark grey and light grey boxes, respectively. (B) Amino acid alignment of *S. cerevisiae* RTS1 and *F. verticillioides* Ppr2. Identical amino acids and similar amino acids are highlighted with dark grey and light grey boxes, respectively.

Fig. S2 Southern analyses of gene deletion mutations. For *PPR1* deletion (A) and *PPR1* re-knockout (B), DNA samples were digested with *EcoRV* and followed the standard protocol for Southern blotting. We anticipated 0.6- and 1.4-kb bands in the wild-type samples. In the mutant, 1.4- and 3.2-kb bands were indicative of a homologous recombination event. For *PPR2* deletion in the SF41 strain (C) and *PPR2* deletion in the wild-type strain (D), DNA samples were digested with *NcoI* and followed the standard protocol for Southern blotting. Wild-type samples showed an 11.6-kb band, whereas the deletion mutants showed a 3.3-kb band. For *PPR1/PPR2* double deletion (*PPR1* deletion in the Δ ppr2 mutant background) (E), DNA samples were digested with *EcoRV* and followed the standard protocol for Southern blotting. We anticipated 0.6- and 14-kb bands in the wild-type samples, whereas, in the double mutant, 1.4- and 3.2-kb bands were indicative of a homologous recombination event. The probes used for these Southern analyses and predicted restriction maps are presented in Fig. 1.

Fig. S3 Deletion mutant phenotypes. (A) Putative *PPR2* deletion mutants (p4, p20 and p29) on V8 agar compared with that of the wild-type (WT) strain. (B) Comparison of Δ ppr1 and rp160 (*PPR1* re-knockout) strains on potato dextrose agar (PDA). (C) Comparison of Δ ppr1 and rp160 (*PPR1* re-knockout) strains on V8 agar. (D) Comparison of Δ ppr2 and *PPR2* re-knockout (pg18 and pg108) strains on V8 agar plates. (E) Comparison of pigment production in potato dextrose broth (PDB) and defined liquid medium (DM).

Fig. S4 (A) Microconidia production per millilitre in wild-type (WT), Δ ppr1, Δ ppr2 and Δ cpr1 strains prior to standardization by unit area. (B) Fumonisin B₁ quantification in wild-type (WT), Δ ppr1, Δ ppr2 and Δ cpr1 strains prior to standardization by the ergosterol production level in the samples. (C) Ergosterol levels in autoclaved corn kernels inoculated with WT, Δ ppr1, Δ ppr2 and Δ cpr1 strains.

Fig. S5 Maize kernel rot assay. (A) Maize seeds (genotype B73) were used to determine the virulence of wild-type (WT), Δ ppr1 and Δ ppr2 strains. Kernel rot was observed under an Olympus SZX12 stereomicroscope system after a 7-day incubation at 25 °C with a 12-h light/12-h dark cycle. At least four biological replicates were performed. Bar, 1 mm. (B) Relative ergosterol levels detected in surface-sterilized corn kernels inoculated with WT, Δ ppr1 and Δ ppr2 strains.

Fig. S6 Localization of Ppr1::GFP and Ppr2::GFP in *Fusarium verticillioides*.

Table S1 Primers used in this study.