Functional characterization of *Fusarium verticillioides* CPP1, a gene encoding a putative protein phosphatase 2A catalytic subunit

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*Fusarium verticillioides* produces the mycotoxin fumonisin B₁ (FB₁) on maize kernels. In this study, we identified a putative protein phosphatase gene CPP1 in *F. verticillioides*, and investigated its role in FB₁ regulation. Previous work has shown that CPP1 expression is elevated in an FB₁-suppressing genetic background. Thus, we hypothesized that CPP1 is negatively associated with FB₁ production. To test this hypothesis, we generated a CPP1 knockout mutant, PP179, and studied the effects of gene deletion on FB₁ biosynthesis and fungal development. PP179 showed elevated expression of FUM genes, and in turn produced higher levels of FB₁ than the wild-type progenitor. Other significant mutant phenotypes included reduced radial growth on agar plates, reduced conidia germination rates, significantly increased macroconidia formation, and hyphal swelling. To verify that these phenotypes were directly due to CPP1 deletion, we complemented PP179 with the wild-type CPP1 gene. The complemented strain PPC4 showed similar expression and FB₁ production to that of the wild-type, providing evidence that CPP1 is negatively associated with FB₁ biosynthesis. Other PP179 phenotypes, such as macroconidiation and hyphal swelling, were also restored to that of wild-type progenitor. Furthermore, we complemented *F. verticillioides* PP179 strain with *Neurospora crassa* wild-type *ppe-1* gene, demonstrating that Cpp1 and PPE-1 proteins are functionally conserved.

Pleiotropic effects of CPP1 deletion led us to hypothesize that CPP1 is associated with multiple downstream signalling pathways in *F. verticillioides*. Identification and functional characterization of downstream Cpp1-interacting proteins are necessary to better understand the complex regulatory mechanisms associated with Cpp1.

**INTRODUCTION**

The fungal pathogen *Fusarium verticillioides* (Sacc.) Nirenburg (teleomorph *Gibberella moniliformis* Wineland) has been the topic of extensive research based on its production of the mycotoxin fumonisin B₁ (FB₁) on maize. FB₁ is a potent carcinogen, and ingestion of fumonisin-contaminated corn has been linked to a variety of illnesses, including leukoencephalomalacia and neural tube defects in animals (Gelderblom et al., 1988; Marasas, 2001; Minorsky, 2002; Missmer et al., 2006). Due to these serious concerns, the US Food and Drug Administration has established guidelines for the fumonisin level in feed and foodstuff (Park & Troxell, 2002). Significant progress has been made in elucidating the fumonisin biosynthetic pathway, and regulatory mechanisms associated with the toxin production. The biosynthetic gene cluster, including the polyketide synthase (PKS) gene FUM1 (Proctor et al., 1999, 2003; Seo et al., 2001), and a number of regulatory genes involved in fumonisin biosynthesis, namely PAC1, FCC1 and ZFR1, have been identified and characterized (Flaherty et al., 2003; Flaherty & Woloshuk, 2004; Shim & Woloshuk, 2001). However, these genes do not show clear epistatic relationships, and therefore it is conceivable that multiple signalling pathways are associated with fumonisin regulation. Moreover, a putative regulatory gene, FUM21, within the FUM cluster, has recently been identified and characterized, but it was shown that the deletion of the FUM21 gene did not completely block fumonisin biosynthesis (Brown et al., 2007). That report suggested that transcriptional regulation of FUM genes can also be affected by putative regulatory gene(s) outside the FUM cluster.

**ABBREVIATIONS:** FB₁ fumonisin B₁; PKS, polyketide synthase; qRT-PCR, quantitative real-time RT-PCR; SSH, subtractive suppression hybridization, WT, wild-type.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of CPP1 is DQ924597.

A description of PP179 complementation using the YEC3 construct (section 1) and a description of how exogenous application of protein phosphatase inhibitor okadaic acid did not induce hyphal swelling in *Fusarium verticillioides* (section 2) are available as supplementary data with the online version of this paper.
cluster. Additionally, the complexity of fumonisin regulation is further enhanced by a variety of physiological and nutritional conditions, notably acidic pH and nitrogen stress, which are known to favour or perhaps trigger fumonisin biosynthesis (Flaherty et al., 2003; Shim & Woloshuk, 1999).

In an effort to identify additional genes associated with fumonisin regulation, Shim & Woloshuk (2001) constructed a subtractive suppression hybridization (SSH) cDNA library to screen genes that are differentially expressed during fumonisin biosynthesis in F. verticillioides wild-type (WT) and FT536 strains. FT536 harbour a mutation in the FCC1 gene, which encodes a type-C cyclin. Mutation of FCC1 results in reduced conidiation and drastic reduction in fumonisin production when grown on maize kernels (Shim & Woloshuk, 2001). Microarray analysis using F. verticillioides oligoarrays has further verified genes that are expressed concomitantly with fumonisin production (Pirttilä et al., 2004). A number of these genes have been selected for characterization of their role in fumonisin biosynthesis. For instance, cDNA encoding a putative zinc binuclear cluster-type transcription factor, later designated ZFR1, has been shown to be positively associated with fumonisin biosynthesis (Flaherty & Woloshuk, 2004). Deletion of ZFR1 in F. verticillioides has been shown to result in greater than 90% reduction in fumonisin production when compared with the WT. Also, Sagaram et al. (2006) have recently demonstrated that GBP1, a gene originally identified in the FT536 SSH cDNA library, encodes a monomeric G-protein that is negatively associated with fumonisin biosynthesis.

In the FT536 SSH cDNA library, we isolated a 300 bp cDNA fragment (ft536_0_M14) that shows a high level of similarity to a Neurospora crassa gene that encodes a probable cell-shape-control protein phosphatase 2A catalytic subunit, PPE-1 (E value, 5e-45). Protein phosphatases catalyse the dephosphorylation of specific substrates that are important for processing various biological and cellular functions (Ceulemans & Bollen, 2004; Dickman & Yarden, 1999; Hunter, 1995), and this catalytic action is known to de-activate signalling pathways induced by a variety of protein kinases (Hunter, 1995). In Arabidopsis, for instance, protein phosphatase type 2C is involved in the negative regulation of the abscisic acid signalling pathway (Saez et al., 2004). Overall, we have limited understanding of the role of protein phosphatases in filamentous fungi, particularly in association with secondary metabolism. Moreover, protein phosphatases in F. verticillioides have not been functionally characterized to date.

Since cDNA ft536_0_M14 was identified among a collection of genes upregulated during fumonisin suppres-
**Table 1.** Primers used in this study

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†qRT-PCR primers for *F. verticillioides* genes homologous to Sac. cerevisiae BNI1, BUD3, BUD6 and SPA2; the *F. verticillioides* genes were designated SepA, RGF, AIP and CPO, respectively.

**F. verticillioides** transformation. *F. verticillioides* protoplasts were generated using the protocol described by Shim & Woloshuk (2001), except that Mureinase (2 mg ml⁻¹) was replaced with Drieselase (5 mg ml⁻¹) (Sigma). The *CPP1* gene disruption vector YEC2 was created by inserting 571 bp DNA from the 5’ region of the *CPP1* gene, and 529 bp DNA from the 3’ region of the *CPP1* gene, into pBP15 vector, which contains a hygromycin phosphotransferase (HPH) gene as a selectable marker (Fig. 1a) (Sagaram et al., 2006). Primers CPP1-A, CPP1-B, CPP1-C and CPP1-D were used to amplify the 5’ and 3’ regions, which were subsequently cloned into pBP15 vector (Fig. 1a). The vector YEC2 was linearized with Nef prior to protoplast transformation. Hygromycin-resistant transformants were selected on regeneration agar medium containing hygromycin (150 μg ml⁻¹), and screened for *CPP1* deletion by PCR and Southern analysis. For PCR, primers (CPP1-che-F, CPP1-che-R and HPH-R2) that provide specific positive and negative amplification were used to detect homologous recombination events (Fig. 1a). For Southern analysis, fungal genomic DNA samples were digested with EcoRI before they were subjected to electrophoresis in a 1% agarose gel. A 500 bp DNA fragment of YEC2, excised by double enzyme digestion (SpeI and SmaI), was labelled with ³²P and used as a probe (Fig. 1a).

The *CPP1* deletion mutant PP179 was complemented with a WT *CPP1* gene fused to a geneticin-resistance gene (GEN). The complementation construct YEC4 was made via single-joint PCR strategy (Sagaram et al., 2006; Yu et al., 2004). Primers CPP1-LC-F and CPP1-LC-R amplified the complete *CPP1* gene plus the 850 bp 5’ untranslated region (UTR) and the 450 bp 3’ UTR. This amplicon was fused with the GEN marker, and the final YEC4 construct was amplified with primers Gene-F and CPP1-LC-R2, using Expand Long Polymerase (Roche). We then transformed YEC4 into PP179

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*Y.-E. Choi and W.-B. Shim*
protoplasts, and screened for colonies resistant to geneticin and hygromycin (Sagaram et al., 2006). The introduction of YEC4 in geneticin-resistant strains was verified by PCR and Southern blot analyses.

**Fumonisin B₁ analysis.** *F. verticillioides* strains were grown on cracked-corn medium for 10 days for fumonisin production analysis. FB₁, the major fumonisin produced by *F. verticillioides*, was extracted and analysed by HPLC, following the method described by Shim & Woloshuk (1999). In addition to FB₁, HPLC analysis, qRT-PCR was used to investigate the relative expression of *FUM1* and other selected PKS genes (Kroken et al., 2003) in the WT and mutant strains. Total RNA samples were prepared from the WT, PP87, PP179, and PPC4 grown on cracked-corn medium for 10 days, and qRT-PCR analysis was performed with SYBR-Green as the fluorescent reporter, using gene-specific primers (FUM1-F and FUM1-R2) for the *FUM1* gene. The expression of each gene was normalized to endogenous *TUB2* gene expression. The gene expression was calibrated using $2^{-\Delta\Delta Ct}$ method (Ct, threshold cycle) (Livak & Schmittgen, 2001); the range of expression was calibrated using $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct}_s - 2^{-\Delta Ct}_a$, where $s$ is the standard deviation of the $\Delta Ct$ value. Subsequently, the total RNA samples of the WT and PP179 strains were subjected to qRT-PCR analysis using gene-specific primers for *FUM1*, *PKS1* (PKS1-F and PKS1-R), *PKS2* (PKS2-F and PKS2-R), and *PKS4* (PKS4-F and PKS4-R). Again, the gene expression was calibrated using the $2^{-\Delta\Delta Ct}$ method, with *TUB2* as the endogenous control.

**Microscopy.** Microscopic observations were made using an Olympus BX51 microscope (Olympus America). A detailed description of features used for imaging from this microscope has been given (Shaw & Upadhyay, 2005). Imaging of hyphal growth phenotypes was performed using an Olympus DP70 camera and DP70-BSW software (version 01.01). Nuclei were stained with Hoechst 33258 dye, as described previously (Shaw & Upadhyay, 2005).

**Complementation of PP179 strain with *N. crassa ppe-1*.** *N. crassa ppe-1* (GenBank accession no. XM_951629) encodes a probable cell-shape-control protein phosphatase. The complementation construct YEC5 was generated by single-joint PCR strategy (Fig. 1a) (Yu et al., 2004). WT *ppe-1* (1.43 kb *ppe-1* gene plus 1.6 kb 5’ UTR and...
RESULTS

F. verticillioides CPP1 encodes a putative protein phosphatase 2A catalytic subunit

The F. verticillioides gene index (The Dana Farber Cancer Institute, http://compbio.dfci.harvard.edu/tgi/fungi.html) and Fusarium group database (Broad Institute of Harvard and MIT, http://www.broad.mit.edu/annotation/fungi/) were screened using the 300 bp EST sequence (ft536_0_M14) for matching cDNA and genomic DNA sequences. The gene index screen resulted in the identification of a 1468 bp tentative consensus 26 726, which contains a 1221 bp ORF. The Fusarium group database search revealed that supercontig 13 in chromosome 1, specifically sequence 301 853 to 303 473 (FVEG_09543), harbours a matching genomic DNA sequence. Taken together, in silico analysis revealed that the CPP1 gene is 1377 bp in length, contains three introns (53, 51 and 49 bp), and is predicted to encode a 407 aa polypeptide.

Sequence analysis of Cpp1 revealed a type 2A protein phosphatase catalytic domain (PP2Ac) between amino acids 32 and 385. This domain is present among a large family of serine/threonine phosphatases. Likewise, Cpp1 displays a high similarity to the PP2Ac domains in a number of eukaryotes, particularly those present in filamentous fungi (Fig. 2). Namely, Cpp1 shares significant similarity with PPE-1 protein, which is a probable cell-shape-control PP2Ac in N. crassa (E value, 0.0) (unpublished; GenBank accession no. XM_951629), and with SitA, which is a PP2Ac involved in the TOR pathway of Aspergillus nidulans (E value, 8e-165) (Fitzgibbon et al., 2005; GenBank accession no. CAG30555). Significantly, these PP2Ac proteins of filamentous fungi are orthologous to the Schizosaccharomyces pombe protein Ppe1, which plays a role in cell morphogenesis and mitosis (Shimanuki et al., 1993). We determined via Northern blot analysis that there is no significant differential expression of CPP1 during the course of fungal growth in DL culture, thereby concluding that CPP1 is a constitutively expressed gene in F. verticillioides (data not shown).

Deletion of CPP1 results in upregulation of FUM1 and overproduction of FB1

F. verticillioides CPP1 gene knockout strains were generated using a double homologous recombination strategy in order to test our hypothesis that CPP1 is associated with FB1 biosynthesis. Of the 243 hygromycin-resistant transformants, two strains, designated PP179 and PP183, were identified in which CPP1 was replaced by the YEC2 construct. Specific primers, designed to produce a PCR amplicon dependent upon homologous recombination, were used to confirm the homologous recombination event. PP179 and PP183 produced the expected band; however, no amplicon was observed for the WT or strain PP87, which is a strain with ectopic YEC2 integration (Fig. 1b). We selected PP179 for further molecular characterization. Southern blot analysis further confirmed that the HPH gene replaced 419 bp DNA within the CPP1 gene. The 571 bp 32P-labelled DNA probe hybridized to a 1.3 kb band in the WT and PP87 strains, whereas it hybridized to a 0.8 kb band in the PP179 strain (Fig. 1c).Northern blot analysis was performed using a 571 bp CPP1 DNA fragment probe, which hybridized to the expected

![Fig. 2. Amino acid alignment of F. verticillioides Cpp1, N. crassa PPE-1 and Asp. nidulans SitA via CLUSTALW. Amino acids common to all proteins are indicated by white letters on a black background. Similar amino acids are indicated by white letters on a grey background. Gaps introduced for alignment are indicated by dashes. Cpp1 protein shares significant similarity with PPE-1 (80% identity and 87% similarity; E value, 0.0) and SitA (70% identity and 78% similarity at the amino acid level; E value, 1e-165).](image-url)
Measurement of the radial growth of *F. verticillioides* strains on 0.2 × PDA plates revealed that PP179 had approximately a 40% reduction in growth rate when compared with the WT progenitor and the PP87 strain (Fig. 3). However, no significant difference in fungal mass (fresh wet weight) was observed when strains were grown on 0.2 × PDB and YPD broth. Subsequently WT, PP87 and PP179 strains were grown on cracked-corn medium and DL medium to test FB1 production. TLC and HPLC analysis revealed that the WT and PP87 did not differ in FB1 production in either cracked-corn medium or DL medium; however, we observed a significant increase in FB1 production in PP179. Specifically, FB1 production on inoculated cracked-corn medium was more than four times greater for PP179 than for the WT (Fig. 4a).

To verify that the growth phenotype and increased FB1 production of PP179 were due to the deletion of *CPP1*, PP179 was complemented using YEC4, which contains a WT copy of the *CPP1* gene fused to *GEN* (Fig. 1a). Following transformation, complemented strains were selected by testing growth on 0.2 × PDA containing hygromycin and geneticin. The putative complemented strain containing YEC4 was designated PPC4. We determined via PCR that PPC4 contained the introduced *CPP1* gene (from YEC4) and the *HYG*-replaced mutant locus (Fig. 1b). Southern analysis verified the PCR data (Fig. 1c). Northern blot analysis of PPC4 showed that *CPP1* gene expression was restored in PPC4 (Fig. 1d). Growth rate reduction and increased FB1 reduction in PP179 were restored to the level of the WT in the PPC4 strain, thus providing further evidence for roles of *CPP1* in growth rate and FB1 production (Fig. 3 and 4a).

**Increased FB1 production in PP179 is due to upregulation of FUM1, the FB1-specific PKS gene**

*FUM1* encodes a PKS that is critical to FB1 biosynthesis (Proctor et al., 1999). Here, we asked the question whether the drastic increase in FB1 production in PP179 is due to altered *FUM1* expression. Total RNA was harvested from WT, PP87, PP179 and PPC4 strains grown on cracked-corn medium, the culture condition used for FB1 production. qRT-PCR analysis of *FUM1* revealed at least 11-fold higher *FUM1* expression in PP179 than in other strains tested (Fig. 4b). Analysis of variance (ANOVA) of the gene expression data from all four strains (*P*<0.01) suggested that *FUM1* expression levels in the WT, PP87 and PPC4 strains were significantly different from that of PP179 strain. When the test was limited to the WT, PP87 and PPC4, the resulting *P* value was 0.862, suggesting that the complementation of PP179 with the *CPP1* gene restored the WT level of *FUM1* expression. The data provide molecular evidence that *CPP1* serves as a negative regulator of FB1 biosynthetic genes in *F. verticillioides*.

As there are over 15 PKS genes in *F. verticillioides* (Kroken et al., 2003), we next investigated the specificity of *CPP1* to regulation of *FUM1*. Kroken et al. (2003) classified 15 type-I PKS genes present in *F. verticillioides* into major clades or subclades. To test the impact of *CPP1* deletion on the expression of PKS genes, we selected four PKS genes from different subclades: *PKS1* (reducing PKS clade II), *PKS2* (reducing PKS clade I), *PKS4* (non-reducing PKS clade I) and *PKS11* (*FUM1*, reducing PKS clade III). qRT-PCR analysis of the four PKS genes revealed that only *FUM1* expression was upregulated significantly in PP179, whereas *PKS1*, *PKS2* and *PKS4* were either downregulated or unchanged (Fig. 4c). The *t* tests for *PKS1*, *PKS2* and *PKS4* expression confirmed no significant difference in expression between the WT and PP179. Our results showed that the major impact of *CPP1* deletion was on *FUM1* expression, suggesting that *CPP1* may have a specific function to regulate *FUM1* in *F. verticillioides*.

**CPP1 is required for hyphal polarity maintenance in *F. verticillioides***

In contrast to the WT and PP87, PP179 showed a distinguishable hyphal swelling when grown in liquid...
medium (Fig. 5a). This phenotype in PP179 became more apparent in the cultures incubated longer than 7 days (data not shown). Nucleus staining was utilized to investigate whether the hyphal-swelling phenotype in PP179 was linked to defective cell-cycle progression. Swollen hyphae of PP179 contained multiple nuclei, whereas nuclei in the WT were uniformly distributed along hyphae (Fig. 5b). This phenotype of PP179 suggested that cell-cycle progression was not affected, but maintenance of hyphal polarity was impaired. Notably, suppression of hyphal swelling in PP179 occurred with the addition of sucrose (1 M) to the medium (Fig. 5c), and this suppression was maintained for up to 10 days. PPC4 showed complete recovery from hyphal swelling demonstrating that this phenotype was due to CPP1 deletion (Fig. 5a).

We next identified well-characterized *Saccharomyces cerevisiae* polarity genes BNI1, BUD3, BUD6 and SPA2 (Amberg et al., 1997; Chant et al., 1995; Sagot et al., 2002; Sheu et al., 1998). Homologues of these genes in filamentous fungi are known to be associated with cell-polarity maintenance and cytokinesis (Harris et al., 1997; Virag & Harris, 2006; Wendland, 2003). We isolated corresponding *F. verticillioides* homologues FVEG_04885.3, FVEG_06243.3, FVEG_11303.3 and FVEG_00700.3, and tested the transcriptional levels in the WT and PP179 strains. qRT-PCR, using gene-specific primers (Table 1), showed no significant difference between the WT and PP179 in expression levels of these genes (data not shown).

**CPP1 is involved in microconidia–macroconidia equilibrium and conidia germination in *F. verticillioides***

One striking feature of PP179 was the high percentage of macroconidia production on V8 agar medium. In *F. verticillioides*, the formation of macroconidia has been shown under a few select conditions, such as UV exposure (Nelson et al., 1983). The WT and PP87 strains produced microconidia, but not macroconidia, on V8 agar. In contrast, approximately 44% of the conidia harvested from PP179 on V8 agar were macroconidia (Fig. 6a). The conidiation profile was the same as the WT in PPC4. We
also observed significant reduction of the conidia-germination rate in PP179. WT and PP87 conidia \((1 \times 10^5)\), when resuspended in DL medium, successfully germinated after 24 h incubation (Fig. 6b). However, under the same conditions, PP179 germination rate was only 30% of that of the WT and PP87 strains (Fig. 6b). The germination deficiency in PP179 was only partially recovered with the complementation by \textit{CPP1} in PPC4 strain (Fig. 6b).

Complementation of PP179 with \textit{Neurospora crassa} \textit{ppe-1} results in phenotypic recovery to WT \textit{F. verticillioides}

Since PPE-1 of \textit{N. crassa} showed the highest similarity to Cpp1, we hypothesized that PPE-1 and Cpp1 are functionally conserved, and that the WT \textit{ppe-1} gene could restore the phenotypic characteristics of the PP179 strain. Complementation construct YEC5, containing WT \textit{ppe-1}
with 5’ and 3’ UTR (Fig. 1a), was transformed into PP179 protoplasts, and the transformants were selected by geneticin and hygromycin resistance. Isolates were further tested by PCR to demonstrate the presence of WT ppe-1. No amplicon of ppe-1 was detected in the WT and PP179 strains, whereas amplicons of the expected size (1.5 kb) were observed in the YEC5-complemented isolates (data not shown). One isolate was selected, and designated ‘NC-com’ strain, and further analysed. Interestingly, the complementation of PP179 with YE5C resulted in restorations of WT morphology, and the reduced growth rate of PP179 was restored to WT level in NC-com (Fig. 3). Notably, the hyphal swelling phenotypes of PP179 were less apparent in NC-com (Fig. 5d). Macroconidia production by PP179 was completely eliminated in NC-com, and germination efficiency was restored to that of WT progenitor (data not shown). Elevated FB1 production in PP179 was significantly reduced in NC-com similar to the WT level (data not shown). These results strongly support the hypothesis that N. crassa PPE-1 and F. verticillioides CPP1 are functionally conserved across the two species.

**DISCUSSION**

In eukaryotic cells, reversible protein phosphorylation provides an important means to regulate various cellular functions in response to external signals. While protein kinases take part in phosphorylation, protein phosphatases catalyse the dephosphorylation of proteins, thereby exerting a fundamental role in regulating cellular processes (Dickman & Yarden, 1999). It has been suggested that about one-third of the eukaryotic proteins are regulated by reversible phosphorylations of specific serine, threonine and/or tyrosine residues (Ceulemans & Bollen, 2004). To date, the major group of serine/threonine protein phosphatases are encompassed by two different structural families. PP1, PP2A, PP2B and PP5 are classified as members of the protein phosphatase P family, while PP2C is a member of Mg\(^{2+}\)-dependent protein phosphatase M family (Dickman & Yarden, 1999). Each protein phosphatase has specific or overlapping roles in regards to regulating cellular functions. Despite their central roles in regulating a wide variety of cellular function, including signal transduction and gene expression, limited information is available on the functions of protein phosphatases in filamentous fungi. In general, fungi have a larger number of protein kinases than protein phosphatases in their genome. For instance, *Sac. cerevisiae* has approximately 6000 genes, of which 113 encode protein kinase genes, but only 31 encode protein phosphatases (Dickman & Yarden, 1999; Sakumoto et al., 1999; Stark, 1996). Considering the deviation in prevalence between protein kinases and protein phosphatases, it is likely that Cpp1 has broad substrate specificity, and interacts with multiple protein kinases in several signalling pathways. Further complicating the process, a 36 kDa PP2A catalytic subunit combines with a 65 kDa regulatory subunit to form a PP2A holoenzyme dimer, with a third variable subunit, known as the B subunit, responsible for substrate specificity (Stark, 1996; Mayer-Jaekel & Hemmings, 1994). Since CPP1 encodes a putative catalytic subunit of PP2A, the broad specificity of substrates of CPP1 may be narrowed with additional protein phosphatase subunit components (Mayer-Jaekel & Hemmings, 1994).

In this study, our main hypothesis was that CPP1 serves as a putative FB1 regulatory gene, perhaps in a negative or suppressive manner. Consistent with our hypothesis, we detected elevated FB1 production in PP179 via TLC and HPLC analyses (Fig. 4a), and subsequent qRT-PCR analysis suggested that this increased FB1 production is a result of an elevated FUM1 expression (Fig. 4b). PKS1 and PKS2 were classified, along with FUM1, as reducing PKSs, whereas PKS4 resided in a separate group of non-reducing PKSs (Kroken et al., 2003). Interestingly, deletion of CPP1 did not have a drastic effect on the expression of other PKS genes, suggesting that the regulatory role of CPP1 is specific to FUM genes and FB1 biosynthesis (Fig. 4c). An expanded transcriptional profiling study is necessary to help us better understand the role of CPP1 in *F. verticillioides* secondary metabolism pathways. Of particular interest is whether CPP1 affects other fumonisin biosynthesis regulatory genes, particularly the genes FUM21, ZFR1 and GBB1 that are positively associated with fumonisin biosynthesis (Brown et al., 2007; Flaherty & Woloshuk, 2004; Sagaram & Shim, 2007). Meanwhile, it is well recognized that type 2A protein phosphatases are associated with cell differentiation and development in eukaryotes, including filamentous fungi (Dickman & Yarden, 1999; Hunter, 1995; Mayer-Jaekel & Hemmings, 1994; Shenolikar, 1994; Yatzkan et al., 1998), and multiple PP179 phenotypes suggested that CPP1 is also involved in multiple downstream signalling pathways in *F. verticillioides*. Strikingly, CPP1 deletion led to a hyphal-swelling phenotype, thereby suggesting the functional role of CPP1 in morphogenesis. We hypothesized that hyphal swelling in PP179 is caused by random deposition of cell wall components, leading to impaired hyphal polarity. In the yeast *Sac. cerevisiae*, mutation in genes BNI1, BUD3, BUD6 and SPA2, which regulate polarity and proper cell development, triggers cell-polarity phenotypes (Amberg et al., 1997; Chant et al., 1995; Sagot et al., 2002; Sheu et al., 1998). While there is no experimental evidence that homologues of these yeast genes are involved in *F. verticillioides* cell-polarity maintenance, studies in other filamentous fungi provide support to our hypothesis. For instance *Asp. nidulans* sepA, the BNI1 homologue, plays a critical role in cytokinesis, and is required for maintenance of polarity during hyphal growth (Harris et al., 1997). In *Asbyya gossypii*, deletion of AgBUD3, the BUD3 homologue, led to a change in actin ring localization, and subsequent aberrant chitin accumulation, ultimately generating delocalized septa (Wendland, 1995). However, our results suggest that hyphal swelling in PP179 is not due to transcriptional defect in *F. verticillioides* BNI1, BUD3, BUD6 and
SPA2-like genes. We are currently investigating putative signalling pathways downstream of Cpp1 involved in maintaining hyphal polarity and growth in *F. verticillioides*.

In *Sac. cerevisiae*, PP2A-related genes have been identified, and designated PPH21, PPH22, PPH3, SIT4 and PPG1 (Zabrocki et al., 2002). A phylogenetic tree containing the well-characterized protein phosphatases in *Sac. cerevisiae* clearly indicates that Cpp1 has the highest homology with SIT4 (76% identity, 84% similarity). SIT4 is a component of the conserved TOR (the target of rapamycin) signalling pathway (Di Como & Arndt, 1996) that interacts with Tap42, the phosphatase regulatory subunit of the TOR pathway, in response to nutrient stress (Cutler et al., 2001). Considering the similarity between SIT4 and Cpp1, we hypothesized that hyphal swelling in PP179 may be caused by the defect in nutritional stress response, and perhaps this process is mediated by *F. verticillioides* Tap42 homologue (FVEG_06413.3) (Fig. 5a, b). The hyphal swelling phenotype in PP179 was particularly apparent in the later stages of growth, perhaps when the fungus is under nutritional stress. In contrast, the hyphal swelling could be reversed in PP179 by osmotic remediation with sucrose (Fig. 5c). In *Sac. cerevisiae*, the protein phosphatase catalytic subunit SIT4 is implicated in G1 to S transition of *ppp-1* (Kosmidou et al., 2001), and this leads us to presume that growth and metabolism and fungal development in *F. verticillioides* Tap42 gene, an amino acid change in PP2A protein (PphA) led to a growth defect, lack of germ tube, and mitotic defect (Kosmidou et al., 2001). In *N. crassa*, the transcript levels of *ppp-1* were increased during hyphal germination and elongation (Zeke et al., 2003). We also speculate that the germination defect in PP179 may be linked to hyphal swelling. The hyphal polarity defect caused by *cpp1* deletion may have initially affected germination, and later led to hyphal swelling.

A report by Yatzkan & Yarden (1999) has suggested a link between PP2A regulatory subunit *rgb-1* and macroconidiation in *N. crassa*: inactivation of *rgb-1* leads to a failure in the formation of mature macroconidia. In contrast, we observed that macroconidia production occurs rarely in *F. verticillioides* under laboratory conditions (Nelson et al., 1983), it is reasonable to presume that Cpp1 plays a role in suppressing macroconidia production in the WT strain. We identified the homologue of *rgb-1* in *F. verticillioides* genome (FVEG_01508.3), and we are currently investigating its role. Moreover, recent characterization of *F. verticillioides* FvVE1 revealed that deletion of this gene results in macroconidiation and growth defect phenotypes similar to those of PP179 (Li et al., 2006). However, the molecular genetic linkage between Cpp1 and FvVE1 has not been determined to date. Taken as a whole, we anticipate that additional proteins, such as other protein phosphatase subunit components and putative Cpp1-interacting proteins, are involved in regulating secondary metabolism and fungal development in *F. verticillioides*. Identification and functional characterization of these proteins will help us unravel the complex regulatory mechanisms associated with Cpp1.

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