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

Yoon-E Choi and Won-Bo Shim

Correspondence

Won-Bo Shim
wbshim@tamu.edu

Department of Plant Pathology and Microbiology, Program for the Biology of Filamentous Fungi, Texas A&M University, College Station, TX 77843-2132, USA

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 *FUM1* expression and FB₁ production similar 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.

Received 9 July 2007

Revised 11 September 2007

Accepted 13 September 2007

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*

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 DQ924537.

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 harbours 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 suppression, we hypothesize that this gene, designated *CPP1* (probable cell-shape-control protein phosphatase), is negatively associated with fumonisin biosynthesis in *F. verticillioides*. To test this hypothesis, we generated a *CPP1* gene deletion mutant, and investigated the role of *CPP1* in

F. verticillioides. In this study, we report that deletion of *CPP1* results in elevated fumonisin production via de-repression (or upregulation) of *FUM1* expression. We also show that mutation in *CPP1* results in pleiotropic phenotypes, suggesting that *CPP1* is associated with multiple signalling pathways that control fungal development and differentiation. Lastly, we demonstrate that *N. crassa* WT *ppe-1* gene can complement *F. verticillioides* *CPP1* gene deletion.

METHODS

Fungal strain, and culture media. *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, University of Missouri, Kansas City, MO, USA) was stored in 20 % (v/v) glycerin at -80 °C. Conidia were produced for inoculum by growing the fungus on V8 juice agar (200 ml V8 juice l⁻¹, 3 g CaCO₃ l⁻¹, and 20 g agar l⁻¹) at 25 °C. For genomic DNA extraction, the fungus was grown in YPD medium (Difco) on a rotary shaker (150 r.p.m.). For RNA isolation, hyphal growth assay, and conidia germination assay, the fungus was grown in defined liquid (DL) medium, pH 6.0 (Shim & Woloshuk, 1999). The medium (100 ml) was inoculated with 1 × 10⁵ conidia, and incubated at 25 °C shaking at 150 r.p.m., under a 14 h light/10 h dark cycle. For the fumonisin assay and total RNA isolation, fungal strains were grown on cracked-corn medium, as previously described (Shim & Woloshuk, 1999). To study colony morphology and growth of fungal strains, we used diluted (0.2 ×) potato dextrose agar (PDA; Difco). For macroconidia and microconidia counts, agar blocks (0.5 cm diameter) were inoculated at the centre of V8 juice agar, and allowed to grow for 7 days. The conidia were harvested in 0.1 % Triton X-100, and counted using a haemocytometer.

Nucleic acid isolation and manipulation. Bacterial plasmid DNA and fungal genomic DNA were extracted by using the Wizard miniprep DNA purification system (Promega) and the OmniPrep genomic DNA extraction kit (G Biosciences), respectively. Total RNA was prepared with Trizol reagent (Invitrogen), or with RNeasy plant mini kit (Qiagen). Southern and Northern analyses were performed as described previously (Sagaram *et al.*, 2006; Sambrook & Russell, 2001). The probes used in all hybridization experiments were labelled with ³²P using Prime-It II random primer labelling kit (Stratagene). DNA sequencing was performed at Gene Technologies Lab (Texas A&M University).

PCR and quantitative real-time PCR (qRT-PCR). All primers used in this study are listed in Table 1. PCR amplifications were performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems). PCR of DNA (except single-joint PCR) was performed in a 25 µl total volume with *Taq* DNA polymerase (Promega). The PCR conditions were 2 min of pre-denaturation at 94 °C, followed by 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 54–57 °C, and 2 min extension at 72 °C, unless specified otherwise. Single-joint PCR was performed using Expand Long Polymerase (Roche) using the manufacturer's suggested protocol.

qRT-PCR analyses were performed in a Cepheid Smart Cycler System with a QuantiTect SYBR Green RT-PCR kit (Qiagen). The concentration of RNA samples was adjusted to 100 ng µl⁻¹, and 1 µl was added to the reaction mix. qRT-PCR amplifications were carried out with 30 min reverse transcription at 50 °C, followed by 15 min pre-denaturation at 95 °C, and 35 cycles of 15 s denaturation at 95 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C. The expression of *F. verticillioides* β-tubulin gene (*TUB2*) (GenBank accession no. U27303) was used as a reference.

Table 1. Primers used in this study

Number	Name	Primer sequence (5'→3')†
1	CPP1-che-F	CAC ATA GAC CTT CCA TTG AAG G
2	HPH-R2	CTG AAA GCA CGA GAT TCT TCG C
3	CPP1-che-R	TGA TCA ATC GTC CTA ATC TCA GG
4	PPE1-che-F1	GTC ATC ACA TCC GAG GAC ATC GAG
5	PPE1-com-R2	CTG GCT CTG GCT ATT TGT TGG ATG TTG G
6	CPP1-A	CTA GAT CAT GGC TTC <u>ACT AGT</u> AGG CGT ACC
7	CPP1-B	CAC TTT GAC TTG ACT <u>CCC GGG</u> TGT TCA CTG
8	CPP1-C	GTG TGG TCA GAT CCA <u>AAG CTT</u> GAA GAT GTC
9	CPP1-D	ACC AAC TCT ACC CAA <u>GGT ACC</u> TCC ATA ACC
13	M13-F	TTG TAA AAC GAC GGC CAG TGA
14	M13-R	CAG GAA ACA GCT ATG ACC ATG
15	CPP1-LC-F	<u>CAT GGT CAT AGC TGT TTC CTG</u> GAT GCC TTC GCA TCC TCC CAG
16	CPP1-LC-R	CCT TCA TTC ACG GCA AGA TGG GTC AAT GG
17	CPP1-LC-R2	CGC TGT TCA AGA GTT GGG CTT TCC
18	Gene-F	GCG AAT TGG AGC TCC ACC GC
19	FUM1-F	ATA ACC ATC TCG GCT CAA CG
20	FUM1-R2	GTA TCT GGT GGA CAA CTT TGG G
21	PKS1-F	GGA TAC ATC TCA ATG GCT GTT GAG G
22	PKS1-R	CCT CAC GAA GAT TAA AGT CGT CG
23	PKS2-F	GGA GTC AGT ATC AGA GTG TAG CTC
24	PKS2-R	CGT CCA TAC TTT GGA GGA GAA CC
25	PKS4-F	GCA TCA AGA CTA AGA TCA ACC AGG
26	PKS4-R	GGC AAT GTA TGC TTC GAG AGC
27	TUB2-F	CAG CGT TCC TGA GTT GAC CCA ACA G
28	TUB2-R	CTG GAC GTT GCG CAT CTG ATC CTC G
29	PPE1-com-F1	CAT GGT CAT AGC TGT TTC CTG CTG TAG TCT TGT GGG CAT CG
30	PPE1-com-R1	GTG ACG ACC AGC GAA TCC CTG GTG
31	RGF-F*	GAC ATC ATC GAG ACA TTA CAG GAT GC
32	RGF-R*	GCT CTT CGT AAG CGA CTA GTC GG
33	SepA-F*	CGA CTC CCG ATA TCA CGA CGT ACT C
34	SepA-R*	GTC GAT ACG GGT GTT ATC CTG GAC
35	CPO-F*	GAC AGG AAC TCC GGG AAC AAC AG
36	CPO-R*	GTC CAC GGT CAT CCA TGA ATC C
37	AIP-F*	CTG CCG AAG ATT CTC CCG TCA G
38	AIP-R*	CAA GGT CAG ATC CTT CGT GGG AG

*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.

†Restriction enzyme sites (underlined) were incorporated in the primers to insert amplicons into pBP15. THE M13-R primer sequence for single joint PCR application is shown in bold (Yu *et al.*, 2004).

***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 *NotI* prior to protoplast transformation. Hygromycin-resistant transformants were selected on regeneration agar medium containing hygromycin (150 µg m⁻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

Fig. 1. Molecular characterization of *F. verticillioides* *CPP1*. (a) WT, schematic representation of the *CPP1* locus in the WT strain. YEC2, the *CPP1* disruption construct, which harbours *HPH* as the selectable marker. PP179, schematic representation of *CPP1* locus in the knockout strain after the homologous recombination event. YEC4, the construct with *CPP1* WT gene fused to *GEN* that was used to complement PP179. YEC5, the construct with *N. crassa ppe-1* gene fused to *GEN* that was used to complement PP179. The left-right arrow on YEC2 indicates the fragment used as the ^{32}P -labelled probe in the Southern blot. The numbered arrows indicate the location of primers used for positive and negative PCR assays (Table 1). S, *SpeI*; S', *SmaI*; H, *HindIII*; K, *KpnI*. (b) PCR analysis of *CPP1* disruption and complementation using the primers described in (a). PP87, ectopic integration strain; PP179 and PP183, *CPP1* knockout strains; PPC3, YEC3-complemented strain (for information on PPC3, see supplementary data, section 1, available with the online version of this paper); PPC4, YEC4-complemented strain. The numbers on the left indicate primer combinations for PCR amplification. (c) Southern blot analysis of transformants. Fungal genomic DNA was digested with *EcoRI*, and the blot was hybridized with ^{32}P -labelled DNA probe [shown in (a)]. Molecular sizes are indicated on the left. (d) Northern blot analysis of the transformants probed with ^{32}P -labelled DNA fragment [shown in (a)] to determine the expression of *CPP1*. Total RNA samples (15 μg) were subjected to electrophoresis in a 1.2% denaturing agarose gel. The gel was stained with ethidium bromide to confirm uniformity of loading (rRNA).

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 $2^{-\Delta\Delta C_T}$ method (C_T , threshold cycle) (Livak & Schmittgen, 2001); the range of expression was calibrated using $2^{-\Delta\Delta C_T - s} - 2^{-\Delta\Delta C_T + s}$, where s is the

standard deviation of the ΔC_T 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 $2^{-\Delta\Delta C_T}$ 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

500 bp 3' UTR) was amplified from the *N. crassa* genomic DNA using the primers PPE1-com-F1 and PPE1-com-R1. These amplicons were fused to *GEN* by joint-PCR using PPE1-com-R2 and Gene-F primers. The final construct, YEC5 was introduced into the protoplasts of PP179, and the transformants were screened for geneticin resistance. The selected isolates were further analysed by PCR, using primers PPE1-che-F1 and PPE1-com-R2 to determine the presence of YEC5 in the genome.

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/fgi/>) 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 26726, 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

Fv Cpp1	-MASGIPPGPANLGFNAGLDEWLEBAKCOYLKERAMKLCERKVEIMMESNIQPVCT	59
Nc PPE-1	--MSTIPKPGPANLRFAGLDEWLEBAKCOYLKEPVMKLCERKVEIMMESNIQPVVT	58
An sitA	MTDSKIPPGPANLKRAGLDEWLEBAKCOYLKESHKLCERKVEIMMESNIQPVST	60
Fv Cpp1	PVTICGDIHQFYDLELFRVGGMPFESNVOAPKTSSTVITSDDIEPPTETITNPLAKK	119
Nc PPE-1	PVTICGDIHQFYDLELFRVGGMPFETNVOAPKTATTVITSDDIEPPTETITDNLAKK	118
An sitA	PVTICGDIHQFYDLELFRVGGMPFASLAEPPKTSVITSDDIEPPTATITDPLAKK	120
Fv Cpp1	IKSSGENATSSAEETEAVEAEAEAEEDFQSTMDQTESGVIVNKSSQSADTRYIFLG	179
Nc PPE-1	IKSSTDSEAGASER-----EPPSEETPOS-----SANTGSQADTRFVFLG	160
An sitA	LGRPGTAGDIDDDDN-----NEAGQKEKSS-----SSSTSEIAVNRNFVFLG	165
Fv Cpp1	DEVDRGYFSLETITLLCLKAKYPDRIVLVRGNHESRQITQVYGFYECCQKYGNASVWK	239
Nc PPE-1	DEVDRGYFSLETITLLCLKAKYPDRIVLVRGNHESRQITQVYGFYECCQKYGNASVWK	220
An sitA	DEVDRGYFSLETITLLCLKAKYPDRIVLVRGNHESRQITQVYGFYECCQKYGNASVWK	225
Fv Cpp1	ACCVDFDLVLAIADGRLCVHGGSLSPERTIDQIRVVARAQEIPEHGAFCDLVWSDPE	299
Nc PPE-1	ACCVDFDLVLAIADGRLCVHGGSLSPERTIDQIRVVARAQEIPEHGAFCDLVWSDPE	280
An sitA	ACCVDFDLVLAIADGRLCVHGGSLSPERTIDQIRVVARAQEIPEHGAFCDLVWSDPD	285
Fv Cpp1	DVETWASPRGAGWLFQDKVADEFNHNGLIARAHQLVNEGYKHFFPENSVVTVWSAP	359
Nc PPE-1	DVETWASPRGAGWLFQDKVADEFNHNGLIARAHQLVNEGYKHFFAEKSVTVWSAP	340
An sitA	DVETWASPRGAGWLFQDKVADEFNHNGLIARAHQLVNEGYKHFFNNNNVVTVWSAP	345
Fv Cpp1	NYCYRCGNVASTMAVDHDDNRFSTFSAVHDDQRHVPANRFGPGDYFLZ	408
Nc PPE-1	NYCYRCGNVASTMDRDNKRFSTFSAVHDDQRHVPAGRFGGQDYFLZ	388
An sitA	NYCYRCGNVASTCEINEDKRFSTFSAVHDDQRHVPGRGGRSYYFLZ	393

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, 8e-165).

1.35 kb transcript band in the WT and PP87, but did not hybridize in PP179 (Fig. 1d).

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 in 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

Fig. 3. Colony morphology and growth of the WT and mutants grown on 0.2 × PDA. (a) Strains were point inoculated with an agar block (0.5 cm diameter), and incubated for 7 days at 25 °C, under a 14 h light/10 h dark cycle. PP87, ectopic integration strain; PP179, *CPP1* knockout strains; PPC4, YEC4-complemented strain; NC-com, YEC5-complemented strain. Growth was significantly impaired in PP179, whereas in PPC4 and NC-com strains, the growth defect was restored to that of the WT progenitor. (b) Radial growth measured and presented as a bar graph. Results are means ± SD of three biological replications.

Fig. 4. FB₁ analysis, and *FUM1* expression. (a) Quantification of FB₁ production in WT, PP87, PP179 and PPC4 strains. Sterile cracked corn (2 g) was inoculated with an agar block (0.5 cm diameter) of WT and mutant strains. After incubation for 10 days at 25 °C, FB₁ was extracted with 10 ml 50 % acetonitrile in water, purified through SPE C18 columns, eluted in 2 ml 70 % acetonitrile in water, and quantified by HPLC. All values represent means (sd) of three biological replications. (b) Expression of *FUM1* gene in WT, PP87, PP179 and PPC4 strains. Total RNA samples were prepared from fungal strains grown in cracked-corn medium for 10 days, and quantitative real-time PCR analysis was performed with SYBR-Green as the fluorescent reporter. The levels of transcription were evaluated using the $2^{-\Delta\Delta C_T}$ method, with *TUB2* as the endogenous control. Data represent the fold differences in gene expression. Three biological replications were performed to obtain standard deviations. (c) Expression analysis of select PKS genes in the WT and PP179. Total RNA samples were prepared from fungal strains grown in cracked-corn medium for 10 days, and qRT-PCR analysis was performed with SYBR-Green as the fluorescent reporter. The levels of transcription were evaluated using the $2^{-\Delta\Delta C_T}$ method, with *TUB2* as the endogenous control. Data represent the fold differences in gene expression. Three biological replications were performed to obtain standard deviations. ●, WT; □, PP179.

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

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

Fig. 5. Hyphal swelling phenotype associated with *CPP1* deletion mutation in *F. verticillioides*. (a) Each strain was inoculated in DL medium with same amount of conidia (1×10^5 conidia mL^{-1}). After incubation for 3 days, photos were taken under the microscope. The arrows indicate hyphal swelling in PP179. The hyphal-swelling phenotype was restored with YEC4 complementation (PPC4). Scale bar, 50 μM . (b) Staining of nuclei was performed with Hoechst 33258 dye in WT and PP179. Multiple nuclei in a swollen single hypha were observed in PP179; this was in contrast to uniformly distributed nuclei in the WT. Scale bar, 10 μM . (c) Suppression of hyphal swelling in PP179 with exogenous sucrose. Sucrose (1 M) was added to the DL medium, and fungal strains (1×10^5 conidia mL^{-1}) were inoculated. The suppression of swelling in PP179 was maintained throughout the 10 day incubation period. WT and PP179 photos were taken on the 10th day. Scale bar, 50 μM . (d) Fungal strains were grown in DL medium for 14 days. Phenotypes of PP179 did not occur in either PPC4 or NC-com. Scale bar, 50 μM .

also observed significant reduction of the conidia-germination rate in PP179. WT and PP87 conidia (1×10^5), 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 *CPP1* in PPC4 strain (Fig. 6b).

Complementation of PP179 with *Neurospora crassa ppe-1* results in phenotypic recovery to WT *F. verticillioides*

Since PPE-1 of *N. crassa* showed the highest similarity to Cpp1, we hypothesized that PPE-1 and Cpp1 are functionally conserved, and that the WT *ppe-1* gene could restore the phenotypic characteristics of the PP179 strain. Complementation construct YEC5, containing WT *ppe-1*

Fig. 6. Macroconidia production, and microconidia germination deficiency, in PP179. (a) WT and PP179 strains were spot inoculated with an agar block (0.5 cm diameter) on V8 juice agar, and incubated for 7 days at 25 °C, under a 14 h light/10 h dark cycle. Conidia were harvested, and quantified with a haemocytometer. The percentage of macroconidia in total conidia is presented in the figure. Three biological replications were performed to obtain standard deviations. Scale bar, 50 mM. (b) Germination efficiency in WT, PP87, PP179 and PPC4 strains was assessed by observing germination of microconidia after 24 h incubation. The percentage germination is shown. Three biological replications were performed to obtain standard deviations. Scale bar, 50 μM .

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 YEC5 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 Mg²⁺-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 *Ashbya 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, 2003). 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 (Jiang, 2006), and this leads us to presume that growth and germination defects in PP179 are the consequence of a defect in cell-cycle progression (Fig. 3 and 6b). In *Asp. nidulans*, 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 macroconidiation was activated in PP179 (Fig. 6a). Since 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.

ACKNOWLEDGEMENTS

We thank Dr Brian D. Shaw for providing expertise on microscopic imaging, and careful reading of this manuscript. Financial support was provided by the USDA-NRI Food Safety Program (2005-35201-16233) and USDA-NRI Interagency Microbial Genome Sequencing Program (2005-35600-16405).

REFERENCES

- Amberg, D. C., Zahner, J. E., Mulholland, J. W., Pringle, J. R. & Botstein, D. (1997). Aip3p/Bud6p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites. *Mol Biol Cell* **8**, 729–753.
- Brown, D. W., Butchko, R. A., Busman, M. & Proctor, R. (2007). The *Fusarium verticillioides* *FUM* gene cluster encodes a Zn(II)2Cys6 protein that affects *FUM* gene expression and fumonisin production. *Eukaryot Cell* **6**, 1210–1218.
- Ceulemans, H. & Bollen, M. (2004). Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol Rev* **84**, 1–39.
- Chant, J., Mischke, M., Mitchell, E., Herskowitz, I. & Pringle, J. R. (1995). Role of Bud3p in producing the axial budding pattern of yeast. *J Cell Biol* **129**, 767–778.
- Cutler, N. S., Pan, X., Heitman, J. & Cardenas, M. E. (2001). The TOR signal transduction cascade controls cellular differentiation in response to nutrients. *Mol Biol Cell* **12**, 4103–4113.
- Di Como, C. J. & Arndt, K. T. (1996). Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev* **10**, 1904–1916.
- Dickman, M. B. & Yarden, O. (1999). Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet Biol* **26**, 99–117.
- Fitzgibbon, G. J., Morozov, I. Y., Jones, M. G. & Caddick, M. X. (2005). Genetic analysis of the TOR pathway in *Aspergillus nidulans*. *Eukaryot Cell* **4**, 1595–1598.
- Flaherty, J. E. & Woloshuk, C. P. (2004). Regulation of fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene, *ZFR1*. *Appl Environ Microbiol* **70**, 2653–2659.
- Flaherty, J. E., Pirttila, A. M., Bluhm, B. H. & Woloshuk, C. P. (2003). *PAC1*, a pH-regulatory gene from *Fusarium verticillioides*. *Appl Environ Microbiol* **69**, 5222–5227.
- Gelderblom, W. C. A., Jaskiewicz, K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vlegaar, R. & Kriek, N. P. J. (1988). Fumonisin – novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl Environ Microbiol* **54**, 1806–1811.
- Harris, S. D., Hamer, L., Sharpless, K. E. & Hamer, J. E. (1997). The *Aspergillus nidulans* *sepA* gene encodes an FH1/2 protein involved in cytokinesis and the maintenance of cellular polarity. *EMBO J* **16**, 3474–3483.
- Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225–236.
- Jiang, Y. (2006). Regulation of the cell cycle by protein phosphatase 2A in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **70**, 440–449.
- Kosmidou, E., Lunness, P. & Doonan, J. H. (2001). A type 2A protein phosphatase gene from *Aspergillus nidulans* is involved in hyphal morphogenesis. *Curr Genet* **39**, 25–34.

- Kroken, S., Glass, N. L., Taylor, J. W., Yoder, O. C. & Turgeon, B. G. (2003). Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *Proc Natl Acad Sci U S A* **100**, 15670–15675.
- Li, S., Myung, K., Guse, D., Donkin, B., Proctor, R. H., Grayburn, W. S. & Calvo, A. M. (2006). *FvVE1* regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in *Fusarium verticillioides*. *Mol Microbiol* **62**, 1418–1432.
- Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.
- Marasas, W. F. O. (2001). Discovery and occurrence of the fumonisins: a historical perspective. *Environ Health Perspect* **109**, 239–243.
- Mayer-Jaekel, R. E. & Hemmings, B. A. (1994). Protein phosphatase 2A – a ‘ménage à trois’. *Trends Cell Biol* **4**, 287–291.
- Minorsky, P. V. (2002). The hot and the classic. *Plant Physiol* **129**, 5–6.
- Missmer, S. A., Suarez, L., Felkner, M., Wang, E., Merrill, A. H., Jr, Rothman, K. J. & Hendricks, K. A. (2006). Exposure to fumonisins and the occurrence of neural tube defects along the Texas–Mexico border. *Environ Health Perspect* **114**, 237–241.
- Nelson, P. E., Marasas, W. F. O. & Toussoun, T. A. (1983). *Fusarium Species; an Illustrated Manual for Identification*. University Park, PA: The Pennsylvania State University Press.
- Park, D. L. & Troxell, T. C. (2002). US perspective on mycotoxin regulatory issues. *Adv Exp Med Biol* **504**, 277–285.
- Pirttilä, A. M., McIntyre, L. M., Payne, G. A. & Woloshuk, C. P. (2004). Expression profile analysis of wild-type and *fcc1* mutant strains of *Fusarium verticillioides* during fumonisin biosynthesis. *Fungal Genet Biol* **41**, 647–656.
- Proctor, R. H., Desjardins, A. E., Plattner, R. D. & Hohn, T. M. (1999). A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. *Fungal Genet Biol* **27**, 100–112.
- Proctor, R. H., Brown, D. W., Plattner, R. D. & Desjardins, A. E. (2003). Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet Biol* **38**, 237–249.
- Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M. P., Nicolas, C., Lorenzo, O. & Rodriguez, P. L. (2004). Gain-of function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signaling. *Plant J* **37**, 354–369.
- Sagaram, U. S. & Shim, W. B. (2007). *Fusarium verticillioides* GBB1, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B₁ biosynthesis and hyphal development but not with fungal virulence. *Mol Plant Pathol* **8**, 375–384.
- Sagaram, U. S., Butchko, R. A. E. & Shim, W. B. (2006). The putative monomeric G-protein GBP1 is negatively associated with fumonisin B₁ production in *Fusarium verticillioides*. *Mol Plant Pathol* **7**, 381–389.
- Sagot, I., Rodal, A. A., Moseley, J., Goode, B. L. & Pellman, D. (2002). An actin nucleation mechanism mediated by Bni1 and profiling. *Nat Cell Biol* **4**, 626–631.
- Sakumoto, N., Mukai, Y., Uchida, K., Kouchi, T., Kuwajima, J., Nakagawa, Y., Sugioka, S., Yamamoto, E., Furuyama, T. & other authors (1999). A series of protein phosphatase gene disruptants in *Saccharomyces cerevisiae*. *Yeast* **15**, 1669–1679.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Seo, J.-A., Proctor, R. H. & Plattner, R. D. (2001). Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet Biol* **34**, 155–165.
- Shaw, B. D. & Upadhyay, S. (2005). *Aspergillus nidulans* *swok* encodes an RNA binding protein that is important for cell polarity. *Fungal Genet Biol* **42**, 862–872.
- Shenolikar, S. (1994). Protein serine/threonine phosphatases-new avenues for cell regulation. *Annu Rev Cell Biol* **10**, 55–86.
- Sheu, Y.-J., Santos, B., Fortin, N., Costigan, C. & Snyder, M. (1998). Spa2p interacts with cell polarity proteins and signaling components involved in yeast cell morphogenesis. *Mol Cell Biol* **18**, 4053–4069.
- Shim, W. B. & Woloshuk, C. P. (1999). Nitrogen repression of fumonisin B₁ biosynthesis in *Gibberella fujikuroi*. *FEMS Microbiol Lett* **177**, 109–116.
- Shim, W. B. & Woloshuk, C. P. (2001). Regulation of fumonisin B biosynthesis and conidiation in *Fusarium verticillioides* by a cyclin-like (C-type) gene, *FCC1*. *Appl Environ Microbiol* **67**, 1607–1612.
- Shimanuki, M., Kinoshita, N., Ohkura, H., Yoshida, T., Toda, T. & Yanagida, M. (1993). Isolation and characterization of the fission yeast protein phosphatase gene *ppe1+* involved in cell shape control and mitosis. *Mol Biol Cell* **4**, 303–313.
- Stark, M. J. R. (1996). Yeast protein serine/threonine phosphatase: multiple roles and diverse regulation. *Yeast* **12**, 1647–1675.
- Virag, A. & Harris, S. D. (2006). Functional characterization of *Aspergillus nidulans* homologues of *Saccharomyces cerevisiae* Sap2 and Bud6. *Eukaryot Cell* **5**, 881–895.
- Wendland, J. (2003). Analysis of the landmark protein Bud3 of *Ashbya gossypii* reveals a novel role in septum construction. *EMBO Rep* **4**, 200–204.
- Yatzkan, E. & Yarden, O. (1999). The B regulatory subunit of protein phosphatase 2A is required for completion of macroconidiation and other developmental processes in *Neurospora crassa*. *Mol Microbiol* **31**, 197–209.
- Yatzkan, E., Szöör, B., Fehér, Z., Dombrádi, V. & Yarden, O. (1998). Protein phosphatase 2A is involved in hyphal growth of *Neurospora crassa*. *Mol Gen Genet* **259**, 523–531.
- Yu, J.-H., Hamari, Z., Han, K.-H., Seo, J.-A., Reyes-Domínguez, Y. & Scazzocchio, C. (2004). Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet Biol* **41**, 973–981.
- Zabrocki, P., Van Hoof, C., Goris, J., Thevelein, J. M., Winderickx, J. & Wera, S. (2002). Protein phosphatase 2A on track for nutrient-induced signaling in yeast. *Mol Microbiol* **43**, 835–842.
- Zeke, T., Kókai, E., Szöör, B., Yatzkan, E., Yarden, O., Szirák, K., Fehér, Z., Bagossi, P., Gergely, P. & Dombrádi, V. (2003). Expression of protein phosphatase 1 during the asexual development of *Neurospora crassa*. *Comp Biochem Physiol B Biochem Mol Biol* **134**, 161–170.

Edited by: J.-R. Xu