Short communication

The Type 2C protein phosphatase FgPtc1p of the plant fungal pathogen *Fusarium graminearum* is involved in lithium toxicity and virulenceLINGHUO JIANG^{1,2,3,*}, JINGRAN YANG¹, FEIYU FAN¹, DAJUN ZHANG¹ AND XULI WANG¹¹State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100094, China²Laboratory of Molecular Microbiology, Tianjin Research Center of Basic Medical Sciences, Tianjin Medical University, Tianjin 300070, China³Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China**SUMMARY**

Type 2C protein phosphatases (PP2Cs) are monomeric protein serine/threonine phosphatases that play various roles in eukaryotic organisms. In this study, we characterized the PP2C encoded by *FgPTC1* in *Fusarium graminearum*, the major causal agent of *Fusarium* head blight on wheat and barley. We found that deletion of *FgPTC1* delays the mycelium growth of *F. graminearum* in response to lithium. Consistently, *FgPTC1* complemented the function of *ScPTC1* in lithium toxicity in *Saccharomyces cerevisiae*. Furthermore, we showed that deletion of *FgPTC1* attenuated the virulence of *F. graminearum* on wheat. Therefore, *FgPTC1* plays an important role in regulating the hyphal growth and virulence of *F. graminearum*.

Fusarium graminearum (*Gibberella zeae*) is the major causal agent of *Fusarium* head blast (FHB) disease on wheat and barley, which causes yield losses and affects the quality of grains (O'Donnell *et al.*, 2000; Windels, 2000). Mycotoxins produced by this fungus strongly affect the safety of human food and animal feed, and FHB is therefore a plant disease of great importance in agriculture (Magan, 2006; Osborne and Stein, 2007). Since the 1990s, an increased frequency of FHB outbreaks has occurred worldwide (Chen *et al.*, 2007; O'Donnell *et al.*, 2004). Moreover, the extensive use of chemical fungicides has caused drug resistance of the pathogen in some parts of China (Chen and Zhou, 2009a, b). The continuous use of fungicides may increase the potential risk of an FHB outbreak. Recent studies on *F. graminearum* have focused on its pathogenesis (Bluhm *et al.*, 2007; Cuomo *et al.*, 2007; Goswami *et al.*, 2006; Ramamoorthy *et al.*, 2007).

The reversible phosphorylation of proteins, controlled by protein kinases and protein phosphatases, is a critical mechanism by which eukaryotic organisms regulate signal transduction pathways. Type 2C protein phosphatases (PP2Cs) are monomeric enzymes that specifically dephosphorylate serine/threonine residues. PP2C activity requires the metal cations Mn^{2+} or Mg^{2+} (Cohen, 1989; Jiang *et al.*, 2001). In *Saccharomyces cerevisiae*, *ScPTC1* encodes a PP2C that inactivates the osmosensing mitogen-activated protein kinase (MAPK) cascade by dephosphorylating Hog1p (Warmka *et al.*, 2001). Deletion of *ScPTC1* delays mitochondrial inheritance and causes defects in tRNA splicing, sporulation and cell separation (Maeda *et al.*, 1993; Roeder *et al.*, 1998). In addition, deletion of *ScPTC1* causes yeast cells to be sensitive to cell wall damage, high calcium and alkaline pH (Gonzalez *et al.*, 2006). Recent studies have shown that ScPtc1p regulates the inheritance of the vacuole and the endoplasmic reticulum during cell division (Du *et al.*, 2006; Jin *et al.*, 2009). Furthermore, ScPtc1p is important for normal functions of the yeast TOR pathway (Gonzalez *et al.*, 2009). Therefore, ScPtc1p is an important regulator for various cellular processes in *S. cerevisiae*.

The genomic sequencing of *F. graminearum* has made it feasible to study the functions of key regulatory genes in signalling pathways through reverse genetics. Using ScPtc1p as query, we identified one single sequence homologue of *ScPTC1*, *FgPTC1* (FGSG_04111.3), in the genome of *F. graminearum*, which encodes a protein of 577 amino acids (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum). FgPtc1p shares 62%, 61%, 58%, 57%, 30% and 25% sequence identities with its homologues in *Podospora anserina* (GENBANK accession no. XP_001907994), *Neurospora crassa* (GENBANK accession no. XP_956698), *Sclerotinia sclerotiorum* (GENBANK accession no. XP_001598439), *Magnaporthe grisea* (GENBANK accession no. XP_359570), *Ustilago maydis* (GENBANK accession no. XP_757538) and *S. cerevisiae*, respectively. Therefore, Ptc1p is conserved in pathogenic and

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nonpathogenic fungi. *FgPTC1* contains one single intron and has an open reading frame (ORF) of 1734 bp. In order to examine whether the protein encoded by *FgPTC1* is a PP2C, we extracted total RNA from log-phase-growing *F. graminearum* mycelia with TRIzol reagent (Invitrogen, Beijing, China), and prepared cDNA with oligo-d(T)₁₆ with MuLV Reverse Transcriptase (Invitrogen). We amplified a 978-bp cDNA fragment, encoding the putative catalytic domain (D133 to V458) of FgPtc1p, with primers FgPTC1H6-F (5'-CGGATCCGAGACTCTATGCCCGACCCAAATC-3'; *Bam*HI site in italic) and FgPTC1H6-R (5'-CCAAGCTTCAAACGACCATAACAAGACAAGTTATC-3'; *Hind*III site in italic). This fragment was cloned into the *Bam*HI and *Hind*III sites of the bacterial vector pET28c (Jiang *et al.*, 2002b), which generated a His₆-FgPTC1C construct expressing an N-terminally His₆-tagged FgPtc1p catalytic domain. The construct was confirmed by DNA sequencing. We induced and purified the recombinant protein in *Escherichia coli* as described previously (Jiang *et al.*, 2001). His₆-FgPTC1C migrated as a 40-kDa protein, as expected in 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and was purified to be nearly a single protein band by immobilization on Ni²⁺-nitrilotriacetate agarose beads and elution from the beads (Fig. 1A).

We examined the PP2C activity of His₆-FgPTC1C proteins using a serine/threonine phosphatase assay system (Promega, Madison, WI, USA). We found that the purified proteins exhibited Mg²⁺- or Mn²⁺-dependent dephosphorylation activity on a synthetic phosphopeptide [RRA(pT)VA] (Fig. 1B). This dephosphorylation activity was significantly inhibited by the general protein serine/threonine phosphatase inhibitor NaF at a concentration of 50 mM, but not by okadaic acid and vanadate (Fig. 1B; data not shown). Taken together, these results suggest that His₆-FgPTC1C indeed possesses the characteristics of a typical PP2C.

To examine the functions of *FgPTC1* in *F. graminearum*, we constructed two deletion constructs for *FgPTC1*, p5'+YG and pHY+3', with the two split-marker plasmids pYG and pHY (gifts from Dr B. Gillian Turgeon, Cornell University, Ithaca, NY, USA; Oide *et al.*, 2006). The 5' end 554-bp flanking fragment of the deleted *FgPTC1* ORF region was amplified with primers FgPTC1-D5F (5'-CTACATAACTACAGAGCTCG-3'; *Sac*I site in italic) and FgPTC1-D5R (5'-AGCAGACTTCCTCCAGTC-3'), and cloned into the pMD18-T vector, yielding pMD5. This fragment was excised with *Sac*I enzyme and subcloned into the *Sac*I site of pYG, yielding p5'+YG. Similarly, the 3' end 464-bp flanking fragment was amplified with the primers FgPTC1-D3F (5'-CGGAGCAAAAGAACTG-3') and FgPTC1-D3R (5'-AGCTCCTATCGAAGCTGCAG-3'), and cloned into pMD18-T, yielding pMD3. This fragment was excised with *Bam*HI and *Hind*III, and subcloned into pHY, generating pHY+3'. The correct orientations of inserts in pMD5, pMD3, pHY+3' and p5'+YG were confirmed by diagnostic restriction digestion and polymerase chain reaction (PCR). The two split-marker constructs, p5'+YG and pHY+3', were linear-

Fig. 1 Expression, purification and activity assay of the His₆-FgPTC1C protein. (A) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of His₆-FgPTC1C proteins. Lanes 0, 30 and 60 in the left-hand gel represent bacterial lysates of cells expressing His₆-FgPTC1C induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 0, 30 and 60 min, respectively. The right-hand gel shows purified His₆-FgPTC1C proteins (2 µg). The gel was stained with Coomassie blue. The molecular mass (kDa) is indicated to the left of the gel. (B) Dependence of the phosphatase activity of His₆-FgPTC1C on cation Mg²⁺ or Mn²⁺. E and S represent His₆-FgPTC1C proteins and the peptide substrate, respectively. The phosphatase activity was determined with purified His₆-FgPTC1C proteins (1 µg) at 30 °C for 30 min.

ized with *Pst*I and *Kpn*I, respectively, and mixed together to transform the protoplasts of *F. graminearum*, as described previously (Hou *et al.*, 2002). Hygromycin B-resistant transformants were isolated and purified by the single-spore method, and their genotypes were examined by PCR with primers FgPTC1-DF (5'-CATCTTTGCGAGGCTCTCG-3'), FgPTC1-F1 (5'-CCAAGCTTATCATCCATCAGGTACG3'; *Hind*III site in italic) and FgPTC1-F4 (5'-CTGCAGCTTCGATAGGAGCT-3'), as exemplified with one transformant in Fig. 2. To generate the revertant of the *fgptc1*

Fig. 2 Replacement of the *PTC1* open reading frame with the hygromycin B-resistant gene (HyG) cassette. (A) Deletion strategy is illustrated in the diagram. (B, C) Polymerase chain reaction (PCR) confirmation for the genotype of the *fgptc1* deletion mutant. PCR templates in lanes 1 and 6 are genomic DNA of the deletion mutant, and PCR templates in lanes 2 and 5 are genomic DNA of the wild-type PH-1 strain. Lanes 1 and 2 represent PCR amplifications with the primers F1 and F4, and lanes 5 and 6 represent PCR amplifications with primers dF and YG. Lanes 3 and 4 represent nest-PCR with primers F1 and YG using the PCR products in lanes 1 and 2 as template DNA, respectively. The sizes of three bands in the DNA marker (M) are indicated to the right. (D) Deletion of *FgPTC1* attenuates the virulence of *Fusarium graminearum* on wheat coleoptiles. This assay was carried out with two independent transformed *F. graminearum* strains for both the deletion mutant (MU) and the revertant (REV). Representative photographs of infected wheat coleoptiles are shown. The brown lesions on the coleoptiles infected with the wild-type (WT), the *fgptc1* deletion mutant and the revertant are indicated with arrows. CK, coleoptiles inoculated with distilled water.

deletion mutant, we first subcloned the 2.1-kb *Xba*I DNA fragment, containing the neomycin phosphotransferase (G418 resistant) gene in pSM334 (a gift from Dr Jin-rong Xu, Purdue University, West Lafayette, IN, USA), into the *Xba*I site of pUC19, generating pUC19-G418. We then amplified the 3-kb *FgPTC1* fragment, containing its promoter, ORF and terminator sequence, with primers FgPTC1-F1 and FgPTC1-F4 and cloned it into pMD18-T. This fragment was confirmed by DNA sequencing, excised with *Hind*III and subcloned into pUC19-G418, yielding

pUC19-G-FgPTC1. pUC19-G-FgPTC1 DNA was linearized with *Sac*I, which has a restriction site at the promoter region of *FgPTC1*, and transformed into the *fgptc1* deletion mutant to obtain G418^R revertants, whose genotypes were confirmed by PCR and their resistant phenotypes to G418 or hygromycin B (data not shown).

Next, we examined the phenotypes for the deletion of *FgPTC1*. We found that the deletion of *FgPTC1* caused the mycelium growth of *F. graminearum* to become sensitive to 0.2 M LiCl but not to 1 M KCl and 1 M NaCl (Fig. 3A). However, deletion of *FgPTC1* did not affect the size (length and width) of conidia of *F. graminearum* induced for up to 9 days in carboxymethyl cellulose (CMC) medium at 25 °C (data not shown). The introduction of *FgPTC1* back reversed the sensitive phenotype of the *fgptc1* deletion mutant to LiCl (Fig. 3B). In liquid potato dextrose agar (PDA) medium containing 0.1 M LiCl, hyphal development of the *fgptc1* mutant was delayed by about 24 h in comparison with the wild-type and the revertant strains (Fig. 3C). These results are consistent with the role of *ScPCT1* in lithium toxicity in *S. cerevisiae* (Ruiz *et al.*, 2006). We also examined the effect of the deletion of *FgPTC1* on the virulence of *F. graminearum* with the wheat coleoptile method (Wu *et al.*, 2005). We found that, at 7 days post-inoculation, the lengths of brown lesions reached 22.0 ± 2.4 mm, 12.7 ± 1.8 mm and 21.2 ± 2.1 mm on wheat coleoptiles infected with the wild-type, the *fgptc1* deletion mutant and the revertant, respectively (Fig. 2C). This result indicates that the deletion of *FgPTC1* attenuates the virulence of *F. graminearum* on wheat.

To determine whether *FgPTC1* could complement the functions of *ScPCT1* in *S. cerevisiae*, we amplified the 1734-bp cDNA of the *FgPTC1* ORF with the primers FgPTC1(ORF)F (5'-GAAGATCTATGTTTGGCGGCAGTTCC-3'; *Bgl*II site in italic) and FgPTC1(ORF)R (5'-GAAGATCTTTATTCCTGCCAGGAGGTG-3'), and cloned it into the yeast vector YPB-ADHpt (Jiang *et al.*, 2002a), yielding YPB-ADHpt-FgPTC1. We found that YPB-ADHpt-FgPTC1 could reverse the sensitivity of yeast *scptc1* deletion cells to 0.4 M LiCl to the level of wild-type BY4741 cells (Fig. 3D; Ruiz *et al.*, 2006). YPB-ADHpt-FgPTC1 also reversed the sensitivity of yeast *scptc1* cells to the cell wall damage agent Congo red (200 µg/mL), as well as to 0.4 M CaCl₂ and 5 mM ZnCl₂, albeit not to alkaline pH (Fig. 3E; data not shown). Therefore, *FgPTC1* is a functional homologue of *ScPCT1*.

In conclusion, we have demonstrated that FgPtc1p is a PP2C and can complement the function of ScPtc1p in lithium toxicity. Consistently, we found that *FgPTC1* is involved in the resistance of mycelium growth of *F. graminearum* to lithium toxicity. Development alterations caused by lithium in *Dictyostelium*, *Drosophila* and *Xenopus*, as a result of its inhibition of glycogen synthase kinase-3β, which regulates cell fate determination in these organisms, have been reported previously (Gould and Manji, 2005; Klein and Melton, 1996; Stambolic *et al.*, 1996). As the primary

Fig. 3 Deletion of *FgPTC1* causes the mycelium growth of *Fusarium graminearum* to become sensitive to lithium. (A) Mycelium growth of the wild-type (WT) PH-1 and three independent deletion mutants for *FgPTC1* on plates containing different salts as indicated. (B) Mycelium growth of PH-1, the deletion mutant (*fgptc1*) and the revertant (*fgptc1::FgPTC1*) on plates containing 0.1 M LiCl. Plates were incubated at 28 °C for 2 days. (C) Conidia of the WT and the *fgptc1* mutant were inoculated in liquid potato dextrose agar (PDA) with or without 0.1 M LiCl, grown at 28 °C and observed for hyphal development. Assays in (B) and (C) were carried out with two independent transformed *F. graminearum* strains for both the mutant (*fgptc1*) and the revertant (*fgptc1::FgPTC1*). (D) *Saccharomyces cerevisiae* wild-type (*ScPTC1*) strain containing pADH1pt vector and its isogenic *scptc1* mutant strain containing pADH1pt or pADH1pt-*FgPTC1* were streaked onto yeast peptone dextrose (YPD) plates with or without 0.4 M LiCl as indicated, and grown at 28 °C for 4 days and 2 days, respectively. (E) *FgPTC1* complements the function of *ScPTC1* in the sensitivity of yeast cells to 200 µg/mL Congo red (CR), 0.4 M CaCl₂ and 5 mM ZnCl₂. The wild-type (*ScPTC1*) strain containing pADH1pt and its isogenic *scptc1* strain containing pADH1pt or pADH1pt-*FgPTC1* were grown in liquid Synthetic dropout medium lacking uracil (SD-URA) (Jiang *et al.*, 2004) at 28 °C overnight, serially diluted 10 times and spotted onto YPD plates without or with the chemical compounds, as described previously (Ruan *et al.*, 2007).

inoculum of FHB disease, the sexual ascospores land on and infect susceptible parts of the host plant. This fungus then produces asexual macroconidia that can overwinter in the soil, where they may have to adapt to a lithium-stress environment through FgPtc1p before they can give rise to mycelia in the next season. Furthermore, we have observed that the deletion of *FgPTC1* attenuates the virulence of *F. graminearum* on wheat. A recent study has shown that the deletion of one of the seven PP2C genes, *CaPTC1*, significantly reduces the virulence of the human yeast pathogen *Candida albicans* (Fan *et al.*, 2009; Hanaoka *et al.*, 2008). Therefore, FgPtc1p could be a potential drug target for the control of FHB. These studies provide a basis for further investigations of the mechanisms regulating the growth, development and virulence of *F. graminearum* through FgPtc1p.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (973) (2006CB101907).

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