FgTep1p is linked to the phosphatidylinositol-3 kinase signalling pathway and plays a role in the virulence of *Fusarium graminearum* on wheat

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SUMMARY

Both mammalian tensin-like phosphatase 1 (TEP1; also known as phosphatase deleted on chromosome 10 (PTEN) or mutated in multiple advanced cancer 1 (MMAC1)) and *Saccharomyces cerevisiae* ScTep1p are involved in the phosphatidylinositol pathway. In this study, we identified the *Fusarium graminearum* locus FGSG_04982.3 (named *FgTEP1*) as the functional homologue of *ScTEP1* in the sensitivity of *S. cerevisiae* cells to wortmannin, the phosphatidylinositol-3 kinase inhibitor. Deletion of *FgTEP1* causes *F. graminearum* mycelial growth to become sensitive to lithium and reduces the production of conidia. Although conidia lacking *FgTEP1* germinate normally, they show reduced germination efficiency in the presence of wortmannin. In addition, we showed that deletion of *FgTEP1* reduces the virulence of *F. graminearum* on wheat. These results indicate that FgTep1p is linked to the phosphatidylinositol-3 kinase signalling pathway in this plant fungal pathogen.

INTRODUCTION

Tensin-like phosphatase 1 (TEP1), also known as phosphatase deleted on chromosome 10 (PTEN) or mutated in multiple advanced cancer 1 (MMAC1), is a human major tumour suppressor and is related to the genesis of breast, endometrial, brain and prostate cancers (Li and Sun, 1997; Steck *et al.*, 1997). Inactivation of one copy of *TEP1* increases the development of tumours, and its homozygous deletion causes embryonic lethality in mice (Di Cristofano *et al.*, 1998; Podsypanina *et al.*, 1999; Suzuki *et al.*, 1998). Deletion of *TEP1* causes the accumulation of the phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃],

which, in turn, activates protein kinase B (PKB/Akt) and induces cellular transformation in embryonic fibroblasts and tumour cells taken from knockout mice (Cantley and Neel, 1999). *TEP1* is also essential in *Drosophila*, but not in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Gil *et al.*, 1999; Heymont *et al.*, 2000; Huang *et al.*, 1999; Ogg and Ruvkun, 1998). TEP1 plays a negative role in the regulation of signalling through the insulin/phosphatidylinositol-3 kinase (PI3K)/Akt pathway that controls dauer formation during larvae development and longevity in *C. elegans* (Masse *et al.*, 2005; Solari *et al.*, 2005). Both lithium and wortmannin have been shown to affect cell growth by inhibiting enzymes, including PI3K and PI4K, in the PI3K signalling pathway in mammalian and fungal cells (Cutler *et al.*, 1997). Yeast cells deleted for *TEP1* are resistant to lithium and wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor, and have a defect in the developmental process of sporulation (meiosis) (Heymont *et al.*, 2000).

Fusarium head blight (FHB; also known as scab) is a devastating disease of wheat, barley and other small grain cereals, and outbreaks in Europe, America, Canada and China during the 1990s have made FHB a global problem (O'Donnell *et al.*, 2004). FHB epidemics in Europe and North America have resulted in huge economic losses as a result of reduced yields, mycotoxin contamination and poor seed quality (Windels, 2000). Mycotoxin contamination of wheat and corn caused by FHB threatens food safety and is of global concern (Bennett and Klich, 2003). *Fusarium graminearum* (teleomorph *Gibberella zeae*) is the primary causal agent of FHB (O'Donnell *et al.*, 2000). Recent studies on *F. graminearum* have focused on its pathogenesis and toxin biosynthesis (Bluhm *et al.*, 2007; Cuomo *et al.*, 2007; Desjardins and Proctor, 2007; Goswami *et al.*, 2006; Ramamoorthy *et al.*, 2007; Yuen and Schoneweis, 2007). In this study, we identified one single homologue of PTEN/MMAC1/TEP1, *FgTEP1*, in the genome of *F. graminearum* and analysed the functions of *FgTEP1*. *F. graminearum* mycelia deleted for *FgTEP1* grow

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normally, but display sensitivity to lithium and show a reduced production of conidia. In addition, deletion of *FgTEP1* reduces the germination of conidia in the presence of wortmannin and renders *F. graminearum* less virulent on wheat.

RESULTS AND DISCUSSION

Through a BLAST search at the *Fusarium* Comparative Database site (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) using the amino acid sequence of *S. cerevisiae* ScTep1p as a query, we identified the locus FGSG_04982.3 (or FG04982.1) as the *F. graminearum* homologous sequence for ScTep1p, and named it FgTep1p. Tep1p is also conserved in two other *Fusarium* species, *F. verticillioides* (FVEG_06752.3) and *F. oxysporum* (FOXG_09154.2). *FgTEP1* has one intron and encodes a protein of 558 amino acids, sharing 85%, 84% and 26% identities with FvTep1p, FoTep1 and ScTep1p, respectively. Similar to ScTep1p, FgTep1p is predicted to have a protein tyrosine phosphatase, catalytic (PTPc) domain by the SMART program (<http://smart.embl-heidelberg.de>).

A previous study has shown that *S. cerevisiae* homozygous *tep1/tep1* deletion cells are resistant to wortmannin, the PI3K inhibitor (Heymont *et al.*, 2000). To determine whether *FgTEP1* could complement the function of the yeast *ScTEP1* gene, we introduced the YPB-ADHpt vector into yeast wild-type (WT) BY4743 and isogenic *tep1/tep1* deletion mutant cells, and introduced the ADHpt-FgTEP1(ORF)-5 plasmid into *tep1/tep1* mutant cells. Consistent with the previous observation on the deletion of *ScTEP1* in another genetic background of the W303A diploid (Heymont *et al.*, 2000), the homozygous *tep1/tep1* mutant showed much better growth in the presence of 20 μ M wortmannin in comparison with its isogenic WT BY4743 strain (Fig. 1A). However, introduction of the YPB-ADHpt-FgTEP1(ORF)-5 plasmid into the *tep1/tep1* mutant led to recovery of its wortmannin sensitivity to the level of the WT BY4743 strain (Fig. 1A). In contrast, no growth difference was observed between these three strains in the absence of wortmannin (data not shown). These results indicate that *FgTEP1* could complement the function of *ScTEP1* in wortmannin sensitivity in yeast cells, suggesting that FgTep1p is a functional homologue of ScTep1p in wortmannin sensitivity. Although FgTep1p and ScTep1p only share 26% identity in overall amino acid sequence, they contain a conserved PTPc domain that is critical for phosphatase activity. This is the case for the *F. graminearum* Type 2C protein phosphatase FgPtc1p, which also shares a limited primary amino acid sequence homology (25%), but a conserved PP2C catalytic domain, with ScPtc1p, and therefore complements the function of ScPtc1p in lithium and calcium sensitivities (Jiang *et al.* 2010).

To examine the functions of *FgTEP1* in *F. graminearum*, we generated the *fgtep1* deletion mutant (*fgtep1*) and the complemented strain (*fgtep1::FgTEP1*). When we measured the diam-

Fig. 1 Functions of *FgTEP1* in yeast and *Fusarium graminearum* cells. (A) Functional complementation of *FgTEP1* for *ScTEP1* in wortmannin sensitivity. The wild-type (WT) *Saccharomyces cerevisiae* BY4743 and the deletion mutant (*tep1/tep1*) strains containing the YPB-ADHpt vector or the ADHpt-FgTEP1(ORF)-5 plasmid were grown at 30 °C in YPD (1% yeast extract, 2% peptone and 2% glucose) medium containing 20 μ M wortmannin, and their growth was monitored by measuring the optical density at 600 nm ($OD_{600\text{ nm}}$) of their cultures 4, 8, 12 and 24 h after inoculation, with an initial $OD_{600\text{ nm}}$ of 0.02. (B) Diameters of colonies of the *F. graminearum* WT PH-1, deletion mutant (*fgtep1*) and complemented strain (CM) (*fgtep1::FgTEP1*) on potato dextrose agar (PDA) plates with or without 0.1 or 0.2 M LiCl at 25 °C for 2 days. (C) Percentages of conidia of the WT PH-1, deletion mutant and complemented strain (CM) germinated 6 and 10 h after incubation in complete medium containing 20 μ M wortmannin. The asterisk (*) shows a statistically significant difference ($P < 0.05$) between the deletion mutant and the WT strain.

eters of the colonies derived from mycelium plugs on potato dextrose agar (PDA) plates, we did not find a significant difference between the WT, deletion mutant and complemented strains (data not shown). However, on PDA plates containing

either 0.1 or 0.2 M LiCl, the colony diameters of the deletion mutant were only one-half of those of the WT and the complemented strain (Figs 1B and 2A). The similar size phenotype was also observed for colonies derived from individual conidia of these three strains on PDA plates containing 0.1 M LiCl (Fig. 2B). The introduction of *FgTEP1* back to the deletion mutant reversed its lithium sensitivity to the level of the WT strain in both cases (Figs 1B and 2A,B). However, no growth difference between the mutant and WT strain was observed on PDA plates containing

Fig. 2 Deletion of *FgTEP1* causes the mycelial growth of *Fusarium graminearum* sensitive to lithium and reduces its virulence on wheat coleoptiles. (A) Growth of the wild-type (WT) PH-1, deletion mutant (*fgtep1*) and complemented strain (CM) (*fgtep1::FgTEP1*) from inoculated plugs on potato dextrose agar (PDA) plates containing 0.1 M LiCl. Plates were incubated at 25 °C for 5 days. (B) Growth of the WT, deletion mutant and complemented strain (CM) from individual conidia on PDA plates containing 0.1 M LiCl. Top row, front view of plates; bottom row, bottom view of plates. Plates were incubated at 25 °C for 5 days. (C) Representative photographs of infected wheat coleoptiles inoculated with conidia of the WT, deletion mutant and complemented strain (CM). The brown lesions on the coleoptiles are indicated with arrows. Coleoptiles inoculated with distilled water (dH₂O) were used as a control.

1 M NaCl or 1 mg/mL carbendazim (MBC) (data not shown), the fungicide used extensively to control FHB in China (Chen *et al.*, 2007). Taken together, these results indicate that deletion of *FgTEP1* increases the sensitivity of mycelial growth to lithium in *F. graminearum*.

Next, we examined whether the deletion of *FgTEP1* had an effect on the production of conidia. After induction for 5 days in carboxymethylcellulose (CMC) medium at 25 °C, the deletion mutant showed a reduced production capacity [$(4.7 \pm 2.1) \times 10^7$ conidia/mL] in comparison with that of the WT [$(9.5 \pm 1.7) \times 10^7$ conidia/mL] and complemented strain [$(9.38 \pm 1.4) \times 10^7$ conidia/mL]. There was no significant difference in the size and morphology of conidia between the deletion mutant and WT strain. The WT strain had an average length of 36.8 ± 4.5 µm and an average width of 4.5 ± 0.3 µm for the three-septa conidia, an average length of 43.2 ± 2.7 µm and an average width of 4.6 ± 0.2 µm for the four-septa conidia, as well as an average length of 44.0 ± 3.4 µm and an average width of 4.6 ± 0.3 µm for the five-septa conidia. The deletion mutant had an average length of 36.3 ± 4.2 µm and an average width of 4.2 ± 0.4 µm for the three-septa conidia, an average length of 44.9 ± 3.3 µm and an average width of 4.8 ± 0.2 µm for the four-septa conidia, as well as an average length of 46.9 ± 3.5 µm and an average width of 4.6 ± 0.3 µm for the five-septa conidia. However, after induction for 9 days in CMC medium containing 0.1 M LiCl at 25 °C, the deletion mutant had an average length of 31.3 ± 4.9 µm and an average width of 4.8 ± 0.1 µm for the two-septa conidia, as well as an average length of 39.6 ± 4.5 µm and an average width of 4.9 ± 0.2 µm for the three-septa conidia. In contrast, the WT strain had an average length of 26.3 ± 4.5 µm and an average width of 4.3 ± 0.6 µm for the two-septa conidia, as well as an average length of 33.8 ± 3.8 µm and an average width of 4.4 ± 0.4 µm for the three-septa conidia. The average length and width of the conidia of the complemented strain were similar to those of the WT strain (data not shown). These results indicate that the loss of *FgTEP1* results in an increased size of *F. graminearum* conidia in the presence of lithium.

We next examined whether the deletion of *FgTEP1* would affect the germination of conidia. Conidia of the deletion mutant germinated normally in liquid complete medium (CM) at 25 °C in comparison with those of the WT and complemented strain (data not shown). However, after 6 h of incubation in CM containing 20 µM wortmannin, the germination rate of the conidia of the deletion mutant was only about 15%, whereas the germination rate of the WT conidia reached 69% (Fig. 1C). Further incubation to 10 h did not increase significantly the germination rate for the conidia of the deletion mutant, whereas the germination rate of WT conidia increased to above 80% (Fig. 1C). This germination defect was rescued by the introduction of the *FgTEP1* gene back to the deletion mutant (Fig. 1C). Taken together, these results

suggest that the deletion of *FgTEP1* reduces the germination efficiency of conidia in the presence of wortmannin in *F. graminearum*.

To examine the function of *FgTEP1* in virulence, we inoculated wheat coleoptiles with conidia of *F. graminearum* strains. The length of brown lesions on coleoptiles infected by the deletion mutant was 4.8 ± 0.5 mm, whereas those infected by the WT strain and the complemented strain showed lesion lengths of 7.8 ± 0.3 mm and 7.4 ± 0.4 mm, respectively (Fig. 2C). In contrast, no brown lesions were formed on wheat coleoptiles inoculated with distilled water (Fig. 2C). These data indicate that the virulence of *F. graminearum* on wheat coleoptiles was significantly attenuated by the deletion of *FgTEP1*.

In conclusion, we have demonstrated that *F. graminearum FgTEP1* is a functional homologue of *ScTEP1* in the sensitivity of yeast cells to wortmannin, the PI3K inhibitor. Although *FgTEP1* is not normally required for the growth of mycelium and conidia, the growth of mycelium and conidial germination were reduced in the presence of wortmannin and lithium, respectively. Our data indicate that *FgTEP1* plays a role in the PI3K signalling pathway in *F. graminearum*, because both lithium and wortmannin have been shown to affect cell growth by the inhibition of enzymes, including PI3K and PI4K, in the PI3K signalling pathway in mammalian and fungal cells (Cutler *et al.*, 1997). It is interesting that *FgTEP1* is not required for asexual development *in vitro*, but is required for the virulence of *F. graminearum* conidia on wheat coleoptiles. It is possible that the lack of *FgTEP1* would disrupt the PI3K signalling of *F. graminearum* during the infection process, which would probably result in attenuated virulence on its host, because it has been shown recently that PI3K signalling is required for virulence of the human fungal pathogen, *Cryptococcus neoformans* (Hu *et al.*, 2008).

In mammalian cells, the *in vivo* substrates of TEP1/PTEN are most probably the lipid phosphatidylinositol phosphates, especially PtdIns(3,4,5)P3 (Furnari *et al.*, 1998; Maehama and Dixon, 1998; Myers *et al.*, 1998). The generation of PtdIns(3,4,5)P3 at the plasma membrane is critical for the regulation of mammalian cell proliferation and survival (Cantley, 2002). However, *S. cerevisiae* and plant cells do not contain detectable amounts of PtdIns(3,4,5)P3 (Bergsma *et al.*, 2001; Dove *et al.*, 1997). Therefore, the physiological substrates for TEP1 in yeast and plant cells could be different from those in mammalian cells. In addition, *ScTEP1* plays a role in the cell wall formation of sexual spores. A *ScTEP1*-G199E mutation, mimicking mammalian PTEN G129E, which causes a loss of catalytic activity on lipid substrates, led to a cell wall defect for sexual spores with the deletion of *ScTEP1*, suggesting that *ScTEP1* might be involved in the dephosphorylation of phosphoinositides other than PtdIns(3,4,5)P3 (Heymont *et al.*, 2000). TEP1 is highly conserved in divergent species of yeast, dimorphic and filamentous fungi (Cid *et al.*, 2008), indicating an important and specialized role of this enzyme in fungal

species. More complex isomers of inositol bis- and trisphosphates are present in filamentous fungi, including *F. graminearum*, than in animal or plant cells (Prior *et al.*, 1993), suggesting that *F. graminearum* might be a good candidate for the elucidation of the *in vivo* substrates for fungal TEP1 enzymes.

EXPERIMENTAL PROCEDURES

Media, strains and chemical reagents

F. graminearum strains were routinely maintained at 25 °C on PDA plates. The *F. graminearum sensu stricto* WT strain PH-1 (NRRL 31084) and the pSM334 plasmid containing the G418 resistance cassette were kindly provided by Dr Jin-rong Xu, Purdue University, West Lafayette, IN, USA. Plasmids pHY (pNLC106) and pYG (pNLC107) were kindly provided by Dr Gillian Turgeon at Cornell University, Ithaca, NY, USA (Oide *et al.*, 2006). *S. cerevisiae* strains were maintained on YPD (1% yeast extract, 2% peptone and 2% glucose) medium. Synthetic dropout (SD) medium was supplemented with appropriate nutrients for plasmid selection and prepared as described previously (Jiang *et al.*, 2004). MBC (98%) was supplied by Shandong Huayang Technology Co. (Ningyang, Shandong, China). MBC stock solution (10 mg/mL) was prepared in 0.1% HCl.

S. cerevisiae BY4743 and its isogenic *tep1/tep1* deletion mutant strains were purchased from Invitrogen, Beijing, China, and maintained on YPD plates at 30 °C. The drug wortmannin (Sigma, Beijing, China) was prepared in dimethylsulphoxide (DMSO) as stock solution (25 mg/mL) and stored at -20 °C. *S. cerevisiae* transformation was performed by the lithium acetate procedure (Ruan *et al.*, 2007).

Molecular cloning

F. graminearum genomic DNA extraction was carried out as described previously (Proctor *et al.*, 1995). To delete the *FgTEP1* gene, we cloned the two DNA fragments (TEP1-a and TEP1-b) that flank the open reading frame of *FgTEP1*. Primers FgTEP1-F1(new) (5'-CAGCGTCGATCTTGATCC-3') and FgTEP1-R1 (5'-TGAAGG TCCAGATCTAGACC-3') were used to amplify the 920-bp fragment TEP1-a, and primers FgTEP1-F2 (5'-TATCCCCACGA TCTCTGTG-3') and FgTEP1-R2 (5'-CCCAAGCTTACTCTCGT CAAAGTCGTC-3', *HindIII* site in italic) were used to amplify the 688-bp fragment TEP1-b from the genomic DNA of *F. graminearum* PH-1 strain (Fig. 3). Both fragments TEP1-a and TEP1-b were cloned into the pMD18-T vector, yielding pMDa and pMDb, respectively. A 480-bp fragment, containing most of TEP1-b, was excised from pMDb with *XbaI*/*SacI* enzymes and subcloned into *XbaI*/*SacI*-digested pMDa, generating pMDab. A 2.1-kb DNA fragment containing the G418 resistance cassette in the pSM334 plasmid was excised with *XbaI* and inserted

Fig. 3 Deletion strategy for *FgTEP1* and polymerase chain reaction (PCR) confirmation. (A) The diagram illustrates the replacement of the *FgTEP1* open reading frame with the G418 resistance cassette in the genome of *Fusarium graminearum*. Primer locations and directions are indicated by horizontal arrows. The restriction sites of *XbaI* are indicated by vertical arrows. IN, intron location in *FgTEP1*. (B–D) PCR confirmation of the genotype of the *fgtep1* deletion mutant. The wild-type (WT) was the *F. graminearum* PH-1 strain.

between the TEP1-a and TEP1-b fragments in pMDab, yielding pMDaG418b, the disruption cassette for *FgTEP1*.

To generate the complemented strain for the *F. graminearum* *fgtep1* deletion mutant, we constructed a plasmid pUC-hph-*FgTEP1* containing a hygromycin B resistance cassette and the *FgTEP1* gene with its own promoter and terminator in the pUC19 vector. First, the 2.1-kb hygromycin B resistance cassette was subcloned into the *XbaI* site of pUC19 from pHY and pYG plasmids, generating pUC-hph. Primers FgTEP1-F1(new) and FgTEP1-R2 were used to amplify the 3.4-kb *FgTEP1* gene from the genomic DNA of *F. graminearum* PH-1 strain (Fig. 3), which was cloned into the pEASY-T1 vector, yielding pEASY-TEP1. The insert sequence in this construct was confirmed by DNA sequencing. The 3.4-kb fragment was excised with *HindIII* enzyme from pEASY-TEP1 and cloned into the *HindIII* site of pUC-hph, yielding pUC-hph-*FgTEP1*.

To clone the *FgTEP1* cDNA, total RNA was extracted from mycelia of the PH-1 strain with Trizol, according to the manufacturer's recommendations. The first-strand cDNA was synthesized with the primer oligo(dT)₁₅ using the Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Invitrogen), according to

the manufacturer's recommendations. We amplified the 1677-bp open reading frame without the intron of *FgTEP1* from its cDNA with primers FgTEP1(ORF)-F (5'-GAAGATCTATGGCGTCTA TCTTGCGAC-3', *BglII* site in italic) and FgTEP1(ORF)-R (5'-GAAGATCTCTACTTCTTGTCCCATC-3', *BglII* site in italic) and cloned it into the pMD18-T vector, yielding pMDTEP1-ORF-20. The inserted DNA sequence in the pMDTEP1-ORF-20 plasmid was confirmed by DNA sequencing before it was subcloned into the *BglII* site of the yeast expression vector YPB-ADHpt (Jiang *et al.*, 2002), which generated the ADHpt-*FgTEP1*(ORF)-5 plasmid expressing *FgTEP1* under the control of the yeast ADH promoter. The insert orientation in ADHpt-*FgTEP1*(ORF)-5 was confirmed by restriction digestion (data not shown).

Disruption of *FgTEP1* and confirmation by polymerase chain reaction (PCR)

We replaced the allele of *FgTEP1* through DNA homologous recombination (Fig. 3A). The disruption cassette pMDaG418b was linearized with *HindIII* enzyme and transformed into protoplasts of *F. graminearum* PH-1. Transformants resistant to 200 µg/mL

G418 were selected and purified through single spore isolation, and their genomic DNA samples were extracted for confirmation of their genotypes by PCR. The 3.7- and 3.4-kb DNA fragments could be amplified from the genomic DNA of the *fgtep1* deletion mutant and WT cells, respectively, with the primer pair FgTEP1-F1(new) and FgTEP1-R2, which was further confirmed by the diagnostic restriction digestion with *Xba*I enzyme (Fig. 3B). The 2.6- and 1.9-kb DNA fragments could be amplified from the genomic DNA of the *fgtep1* deletion mutant, but not from WT cells, with primer pairs FgTEP1-F1 (5'-AAGGAGTACTACG ATGCCTC-3') and G418-F (5'-AACCAAGATGG ATTGCACGC-3'), and G418-R (5'-TGATGCTCTTCGTCCAGATC-3') and FgTEP1-R2, respectively (Fig. 3C). In contrast, the 2.5- and 1.8-kb DNA fragments could be amplified from the genomic DNA of WT cells, but not from the *fgtep1* deletion mutant, with primer pairs FgTEP1-ATG and FgTEP1-R2, and FgTEP1-ATG (5'-CGCGGAT CCGAATGGCGTCTATCTTGCAC-3') and FgTEP1-TAG (5'-ACAAG CTCTACTTCTTGTTCCCATCTTAACC-3'), respectively (Fig. 3D).

To generate the complemented strain, the pUC-hph-FgTEP1 plasmid DNA was linearized with *Bss*HI enzyme, which has a cutting site at the promoter region (within TEP1-a) of *FgTEP1*, and transformed into the protoplasts of the *F. graminearum* *fgtep1* deletion mutant cells. Hygromycin B-resistant transformants were selected, and their genotypes were confirmed by PCR with primer pairs FgTEP1-F1 and FgTEP1-TAG, as well as FgTEP1-ATG and FgTEP1-TAG (data not shown).

Growth assay and production of conidia

For growth assay, 5-mm mycelium plugs cut from the edge of a 3-day-old colony were placed on PDA containing the different reagents tested. The inoculated plates were incubated at 25 °C. Each experiment was carried out with three replicates and repeated three times. For the production of conidia, four 5-mm mycelium plugs were introduced into 100 mL of liquid CMC medium and cultured at 25 °C (Seong *et al.*, 2006). Conidia were harvested by centrifugation at 4000 *g* for 5 min and washed twice with sterile, distilled water. The concentration of conidia was determined with a haemocytometer. To check the effect of LiCl on the production of conidia, liquid CMC medium containing 0.1 M LiCl was used for the production of conidia at 25 °C. The morphology of conidia was observed under an OLYMPUS BH-2 microscope. The width of the middle intercalary cell and the length from the elongated apical cell to the pedicellate foot cell of 100 conidia were measured for each strain examined. CM was used for the germination study on the conidia of each strain as described previously (Seong *et al.*, 2008).

Complementation test

Saccharomyces cerevisiae cells containing appropriate plasmids were maintained at 30 °C on SD–URA (SD lacking uracil) plates.

For wortmannin resistance assay, yeast cells were grown at 30 °C in SD–URA medium overnight, and inoculated with a final optical density at 600 nm (OD_{600 nm}) of 0.02 in YPD medium with or without 20 µM wortmannin. Cells were further incubated with shaking at 30 °C, and OD_{600 nm} values for each strain were measured at 4, 8, 12 and 24 h post-inoculation.

Virulence studies

The virulence of the deletion mutant for *FgTEP1* was examined using the wheat coleoptile method on the wheat variety 'Chinese Spring' (Wu *et al.*, 2005). One hundred wheat coleoptiles were inoculated for the virulence test of each *F. graminearum* strain. Distilled water was used as a negative control. The lengths of brown lesions on coleoptiles were measured after incubation of the inoculated seedlings for 7 days at room temperature in a growth chamber.

Statistical analysis

Statistical analyses were carried out using the statistical package SPSS 10.0 for Windows. Student's *t*-test for paired samples was used for comparison between two groups. *P* values of less than 0.05 were considered to be statistically significant.

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