The TOR signaling pathway regulates vegetative development and virulence in *Fusarium graminearum*

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Abstract

- The target of rapamycin (TOR) signaling pathway plays critical roles in controlling cell growth in a variety of eukaryotes. However, the contribution of this pathway in regulating virulence of plant pathogenic fungi is unknown.
- We identified and characterized nine genes encoding components of the TOR pathway in *Fusarium graminearum*. Biological, genetic and biochemical functions of each component were investigated.
- The FgFkbp12-rapamycin complex binds to the FgTor kinase. The type 2A phosphatases Fgp2A, Fgsit4 and Fgp1 were found to interact with Fgtap42, a downstream component of FgTor. Among these, we determined that Fgp2A is likely to be essential for *F. graminearum* survival, and Fgsit4 and Fgp1 play important roles in cell wall integrity by positively regulating the phosphorylation of Fgmvg1, a key MAP kinase in the cell wall integrity pathway. In addition, the Fgp1 interacting protein, Fgtip41, is involved in regulating mycelial growth and virulence. Notably, Fgtip41 does not interact with Fgtap42 but with Fgp1, suggesting the existence of Fgtap42:Fgp1:Fgtip41 heterotrimer in *F. graminearum*, a complex not observed in the yeast model.
- Collectively, we defined a genetic regulatory framework that elucidates how the TOR pathway regulates virulence and vegetative development in *F. graminearum*.

Introduction

All living organisms interact actively with their surrounding environments and modulate their physiology to maintain cellular homeostasis. This adaptation process is highly coordinated via diverse signaling pathways. The target of rapamycin (TOR) signaling pathway plays a pivotal role in nutrient signal transduction in eukaryotes (Wang & Proud, 2009). Rapamycin was discovered in the early 1970s as an antifungal agent against the pathogenic yeast *Saccharomyces cerevisiae* (Wang & Proud, 2009). Later, it was found to inhibit proliferation of mammalian cells and to possess immunosuppressive properties. These intriguing observations attracted researchers to investigate the mode of action of this compound. In the early 1990s, Tor kinase was first discovered in the budding yeast *Saccharomyces cerevisiae* (Heitman et al., 1991). In *S. cerevisiae*, rapamycin forms a complex with the peptidyl-prolyl cis-trans isomerase Fkbp12 (FK506 binding protein of 12 kDa) (also named Fpr1), and this complex then binds to and inhibits Tor kinases (Heitman et al., 1991). Subsequently, Tor kinases have been identified in various eukaryotes ranging from yeasts to mammals.

In eukaryotic cells, the TOR pathway responds to nutrients and growth factors to orchestrate cell growth and proliferation. Most organisms, including mammals, have a single Tor kinase. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, however, contain two Tor homologs (Sherz et al., 2010), and the protozoans *Leishmania major* and *Trypanosoma brucei* possess three and four TOR-like genes, respectively (Barquilla et al., 2008; Madeira da Silva & Beverley, 2010). The Tor kinases interact with other proteins to form two complexes known as TORC1 and TORC2, which are essential regulators of cell growth in response to nutrients, hormones or stresses in *S. cerevisiae*. TORC1 represents the rapamycin-sensitive signaling branch that mediates temporal control of cell growth by activating anabolic processes such as ribosome biogenesis, protein synthesis, transcription and nutrient uptake, and by inhibiting catabolic processes such as autophagy and ubiquitin-dependent proteolysis. TORC2 is rapamycin insensitive and is required for the actin cytoskeleton organization (reviewed in Wullschleger et al., 2006).

In *S. cerevisiae*, the Tap42-phosphatase complexes are major targets of the Tor kinases in the rapamycin-sensitive signaling pathway (Yan et al., 2006). In these complexes, Tap42 interacts with the catalytic subunits of the type 2A and 2A-like phosphatases, such as Pph3, Pph21, Pph22 and Sit4 (Di Como & Arndt, 1996; Wang et al., 2003). Under starvation or rapamycin...
In a preliminary study, we found that rapamycin is a very potent inhibitor of fungal Tor kinase in plant pathogenic fungi (Beck & Hall, 1999). Tap42-dissociated Sit4 may dephosphorylate the GATA transcription factor Gln3. The dephosphorylated form of Gln3 is dissociated from Ure2, a cytoplasmic anchor protein, and imported into the nucleus (Crespo & Hall, 2002; Inoki et al., 2005; Di Como & Jiang, 2006). In the nucleus, Gln3 binds to GATA-containing promoters and activates transcription of GAP1, MEP1, GLN1 or GDH1 that are required for the adaptation of S. cerevisiae to less preferred nitrogen (N) sources (Cooper, 2002).

Interestingly, genome-wide searches for Tor kinases in filamentous fungi — for example, Aspergillus species, Fusarium graminearum, Neurospora crassa and Magnaporthe oryzae — revealed that all contain only a single Tor ortholog (Shertz et al., 2010). Thus far, little is known about the functions of fungal Tor kinase and the genetic pathways associated with TOR in plant pathogenic fungi (Teichert et al., 2006; Shertz & Cardenas, 2011). Rapamycin is effective against fungi including Cryptococcus neoformans, Aspergillus species and Podospora anserine, as well as the phytopathogenic fungus Botrytis cinerea (Cruz et al., 1999; Demethon et al., 2003; Muthuvijayan & Marten, 2004; Melendez et al., 2009), suggesting that functions of the TOR pathway in plant pathogenic fungi may be similar to that in S. cerevisiae. However, questions remain unanswered on how this pathway regulates and impacts various cellular processes including virulence in plant pathogenic fungi.

Fusarium graminearum (teleomorph: Gibberella zeae) is an economically important plant pathogen that causes Fusarium head blight (FHB) on various cereal crops (Goswami & Kistler, 2004). In addition to yield reduction, mycotoxins produced by F. graminearum in infested grains pose a serious threat to human and animal health (McMullen et al., 1997; Pestka & Smolinski, 2005). In a preliminary study, we found that rapamycin is a very strong inhibitor of F. graminearum growth, prompting us to select this fungus as a model to investigate the functional role of the TOR pathway in plant pathogenic fungi. Thus, we identified putative components of the TOR pathway in F. graminearum (Supporting Information Table S1), and characterized deletion mutants of these genes. Our study shows that the TOR signaling pathway plays important roles in various cellular processes including cell wall integrity, secondary metabolisms and virulence in F. graminearum. Through this study, we established a genetic framework that provides an explanation to how the TOR signaling pathway regulates vegetative differentiation and virulence in F. graminearum.

Materials and Methods

Fungal strains, media and culture conditions

Fusarium graminearum strain PH-1 was used as the wild-type (WT) strain for constructing various gene deletion mutants in this study. The WT strain, resulting mutants and complemented strains were routinely cultured on potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 20 g agar and 1 l water) at 25°C with a 12 h:12 h, light:dark cycle. The WT strain and its derived mutants were grown on carrot agar for induction of sexual development (Klittich & Leslie, 1988) near-UV light (wavelength, 365 nm; HKiv Co., Ltd, Xiamen, China), and in mung bean broth (MBB) for conidiation assays under continuous light. Assays for virulence were performed as described previously (Jiang et al., 2011a). Each experiment was repeated three times.

Isolation of a rapamycin-resistant (RR) mutant of F. graminearum

In order to induce RR mutants, 1 × 10⁵ conidia of PH-1 were spread on PDA plates amended with 10 µg ml⁻¹ rapamycin, and then irradiated under UV for 90 s. After the plates were incubated at 25°C in the dark for 4 d, a RR colony growing on the plate was obtained. A single-spore strain from the resistant colony was further used for DNA sequence analyses of FgKPB12 and FgTOR genes.

Construction of vectors for gene deletion and complementation

The primer pairs used to amplify the flanking sequences or full sequence of each gene are listed in Table S2. Constructs for gene deletion and complementation of F. graminearum were carried out as described previously (Jiang et al., 2011a). PCR products were transformed into PH-1 protoplasts by employing polyethylene-glycol (PEG)-mediated protoplast transformation (Proctor et al., 1995). Putative gene deletion mutants were identified by PCR assays with primer pairs from 45 to 66 as listed in Table S2, and were further confirmed by Southern assays (Fig. S1). All mutants generated in this study were preserved in 15% glycerol at −80°C.

Construction of 3 × FLAG and GFP fusion cassettes

In order to create the FgSIT4-3 × FLAG fusion construct, the FgSIT4 region was amplified with the primers 126 and 127 (Table S2). The resulting PCR products were co-transformed with Xhol-digested pHZ126 (Zhou et al., 2011a) into XK1-25 (Bruno et al., 2004). The FgSIT4-3 × FLAG fusion vector was recovered from yeast transformants and subsequently transformed into the WT strain PH-1. FgPPG1-3 × FLAG, FgTAP42-3 × FLAG and FgTAP42-GFP fusion constructs were constructed using the same strategy.

Microscopic examinations of hyphal and conidial morphology, and GFP fluorescence

The hyphal morphology of each mutant was examined with a Leica TCS SP5 imaging system using fresh mycelia harvested from 3-d-old colonies of each strain growing on PDA plates amended with rapamycin at 0, 0.025, 2.5 or 250 ng ml⁻¹. In addition, hyphal septation was examined after mycelia of each strain were stained with calcofluor white. For histochemical
analysis of lipid droplets in the hyphae, mycelia were mounted in Nile Red staining solution consisting of 20 mg ml\(^{-1}\) polyvinylpyrrolidone and 2.5 mg ml\(^{-1}\) Nile Red Oxazone (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, Sigma) in 50 mM Tris-maleate buffer (pH 7.5) (Jiang et al., 2011b). Following the treatment, lipid droplets fluoresce within a few seconds and can be viewed under a microscope with episcopic fluorescence attachment. To observe nuclei, fresh conidia or mycelia were washed with sterilized water and stained with 10 μg ml\(^{-1}\) 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Calcofluor white staining was carried out as described previously (Rui & Hahn, 2007). For observation of GFP signals, fresh mycelia and conidia were examined with the Zeiss LSM780 confocal microscope (Carl Zeiss AG, Germany).

For conidiation assays, three mycelial plugs of each strain were inoculated in 30 ml MBB supplemented with 0, 0.025, 2.5 or 250 ng ml\(^{-1}\) rapamycin. After incubation in a shaker with 180 rpm at 25°C for 1 wk, conidia of each sample were collected by centrifugation and calculated by a hemocytometer. The experiments were repeated three times.

** Determination of deoxynivalenol (DON) production**

A 50-g aliquot of healthy wheat kernels was autoclaved and then inoculated with five mycelial plugs of each strain. Wheat kernel aliquots inoculated with five agar plugs were used as a negative control. After incubation at 25°C for 20 d, DON and fungal ergosterol were extracted using previously described protocols (Mirocha et al., 1998). The DON extracts were purified with PuriToxSR DON column TC-T200 (Trilogy analytical laboratory), and amounts of DON and ergosterol in each sample were determined using a Waters 1525 HPLC system (Liu et al., 2013). The experiment was repeated three times.

** Yeast strains and complementation assays**

The full-length cDNA of each gene tested was amplified using primer pairs from 89 to 100 as listed in Table S2. The PCR product was digested with appropriate enzymes and cloned into the pYES2 vector (Invitrogen), and then transformed into the corresponding yeast mutant. Yeast transformants were selected on synthetic medium lacking uracil (Clontech, Palo Alto, CA, USA). The pairs of yeast two-hybrid plasmids activation domain vector pGADT7 (Clontech, Mountain View, CA, USA), respectively. The pairs of yeast two-hybrid plasmids were co-transformed into S. cerevisiae strain AH109 following the LiAc/SS-DNA/PEG transformation protocol (Schiestl & Gietz, 1989). In addition, a pair of plasmids, pGBK7T-53 and pGADT7, served as a positive control. The following pairs of plasmids were used as negative controls: pGBK7T-Lam and pGADT7; pGBK7T and pGADT7-FgPP2A; pGBK7T and pGADT7-FgSIT4; pGBK7T and pGADT7-FgPPG1; pGADT7 and pGBK7T-FgTIP41; pGADT7 and pGBK7T-FgTAP42; and pGADT7 and pGBK7T-FgMSG5. Transformants were grown at 30°C for 3 d on synthetic medium lacking Leu and Trp, and then transferred to the medium stripped of His, Leu and Trp and containing 5 mM 3-amino triazole (3-AT) to bind activity (Jiang et al., 2011b). The transformants were also assayed for β-galactosidase activity following the previously published protocol (Zhou et al., 2011b). Three independent experiments were performed.

** Western blotting assay**

Six mycelial plugs of each tested mutant were inoculated into 150 ml potato dextrose broth (PDB) and incubated at 25°C with agitation (200 rpm) for 36 h. Mycelia were harvested, washed with deionized water, and then ground in liquid nitrogen. Approximately 200 mg finely ground mycelia were resuspended in 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM PMSF) and 10 μl of stopwatch inhibitor cocktail (Sangon, Shanghai, China). After homogenization with a vortex shaker, the lysate was centrifuged at 10 000 g for 20 min at 4°C. Then, 100 μl of supernatant was mixed with an equal volume of 2× loading buffer and boiled for 5 min. Subsequently, 15 μl of each sample was loaded onto SDS-PAGE gels. The proteins separated on SDS-PAGE gels were transferred onto a polyvinylidene fluoride membrane with a Bio-Rad electroblotting apparatus. The FgMgv1 kinase and its phosphorylated protein were detected with the PhosphoPlus p44/42 MAP kinase antibody kit (Cell Signaling Technology, Beverly, MA, USA). The horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for antigen antibody detections. The monoclonal anti-FLAG (Abmart, Shanghai, China) was used at a 1:1000–1:2000 dilution for immunoblot analysis. The experiment was repeated three times.

** Affinity purification and mass spectrometry analysis**

Protein extraction was performed as already described. Approximately 50 μl of anti-FLAG agarose (Abmart, Shanghai, China) was added to capture FgTap42, FgSit4 or FgPPg1-interacting proteins, following the manufacturer’s instructions. After incubation at 4°C overnight, the agarose was washed three times with 500 μl of TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). Proteins binding to the beads were immediately eluted with 60 μl of elution buffer (0.2 M glycine, pH 2.5). Eluant was instantly neutralized with 3 μl of neutralization buffer (1.5 M Tris, pH 9.0) and digested with trypsin using a previous described protocol.
Co-immunoprecipitation (Co-IP) assay

FgSIT4 and FgTIP41 were separately amplified and cloned into pHZ126 (Zhou et al., 2011a) by the yeast gap repair approach (Bruno et al., 2004) to generate the 3× FLAG fusion constructs. A similar approach was employed to generate the GFP fusion construct for the pFL1 vector containing FgSIT4 and FgSIT4-FLAG. The resulting fusion constructs were verified by DNA sequencing and transformed into the WT PH-1. Transformants expressing the fusion constructs were confirmed by Western blot analysis. For Co-IP assays, total proteins were isolated and incubated with the anti-FLAG agarose as described above. Proteins eluted from agarose were analyzed by Western blot detection with monoclonal anti-FLAG and monoclonal anti-GFP antibodies (Abmart, Shanghai, China).

Conventional nucleic acid manipulations

The probes for Southern hybridization analyses of the mutants were labeled with digoxigenin (DIG) using the high prime DNA labeling and detection starter kit II according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany).

Results

Rapamycin exhibits a strong inhibition against F. graminearum

Before we investigated the TOR signaling pathway in F. graminearum, we assayed the sensitivity of the WT strain PH-1 to rapamycin. Carbendazim and tebuconazole – two fungicides widely applied for FHB management – were used as controls. Radial growth of PH-1 was severely inhibited on PDA amended with 0.25 μg ml⁻¹ rapamycin (Fig. 1a). Microscopic examination showed that hyphae treated with rapamycin had more branches than the untreated hyphae (Fig. 1b). Interestingly, rapamycin treatment led to increased hyphal septation frequency in F. graminearum (Fig. 1b), indicating that the TOR pathway is involved in the regulation of septum formation or cell wall integrity. In addition, rapamycin-treated hyphae contained many lipid droplets, as visualized by Nile Red staining (Fig. 2a), suggesting that the TOR pathway is involved in the regulation of lipid metabolism in F. graminearum. Rapamycin is also known to induce autophagy in P. anserina and Aspergillus oryzae (Dementhon et al., 2003; Pinan-Lucarre et al., 2005; Kikuma et al., 2006). Because autophagy is involved in nutrient recycling during starvation and plays an important role in growth and development in filamentous fungi (Pollack et al., 2009), we tested whether rapamycin triggers autophagy in F. graminearum. As shown in Fig. 2(b), similar to the autophagy induced by a minimal medium lacking an N source, the autophagy was readily observed in hyphae of F. graminearum treated with 0.25 μg ml⁻¹ rapamycin for 4 h. In addition, rapamycin also exhibited a strong inhibitory effect on asexual reproduction: F. graminearum was unable to produce conidia in MBB amended with 250 ng ml⁻¹ rapamycin (Fig. 2c); with 2.5 ng ml⁻¹ rapamycin, c. 90% inhibition of conidiation was observed. Collectively, these results suggest the likelihood that the TOR pathway plays important roles in vegetative growth and differentiation in F. graminearum.

Deletion of FgFKBP12 leads to resistance to rapamycin in F. graminearum

In S. cerevisiae, rapamycin does not directly target Tor kinase but, rather, binds to Fkbp12. Rapamycin and Fkbp12 form a gain-of-function complex, and this negatively regulates the Tor kinase activity (Heitman et al., 1991). F. graminearum harbors one FKBP12 ortholog (FGSG_09690, named FgFKBP12) encoding a protein with 57% similarity to S. cerevisiae Fkbp12. Using a targeted gene deletion strategy, we generated a FgFKBP12 deletion mutant (ΔFgFKBP12). Morphological analyses showed that mycelial growth of the mutant was comparable to that of the WT progenitor on PDA (Fig. S2a), suggesting that FgFkbp12 is dispensable for hyphal growth. Fungicide sensitivity tests showed that ΔFgFKBP12 confers resistance to rapamycin and FK506 in F. graminearum (Fig. S3), but not to other fungicides including
carbendazim and tebuconazole (data not shown). The sensitivity to rapamycin and FK506 was restored in the complemented strain (ΔFgFKBP12-C) (Fig. S3).

We found two additional genes, FGSG_01408 (named FgFKBP20) and FGSG_01059 (FgFKBP54), encoding proteins with 45% and 49% similarity to Fkbp12. These gene deletion mutants did not show recognizable changes in growth on PDA and in sensitivity to rapamycin (Fig. S2a,b). These results indicate that FgFkbp12, rather than FgFkbp20 and FgFkbp54, is associated with rapamycin toxicity in F. graminearum.

The S1866L mutation in the FgTor kinase confers rapamycin resistance

Saccharomyces cerevisiae has two TOR kinase genes; TOR1 is dispensable, whereas TOR2 is essential (Heitman et al., 1991). However, in silico analysis revealed that FGSG_08133 (FgTor) is the only TOR kinase gene predicted in F. graminearum. To determine its function, we targeted FgTor for gene deletion. We recovered > 80 hygromycin-resistant transformants; however, all of them were ectopic mutants and we failed to retrieve a null mutant. These results suggested that the deletion of FgTor in F. graminearum may be lethal, which is consistent with an earlier report on systematic characterization of the F. graminearum kinome (Wang et al., 2011).

Concurrently, we obtained a RR mutant by UV mutagenesis that grew remarkably better than PH-1 on PDA amended with 10 μg ml⁻¹ rapamycin (Fig. S4a). We amplified and sequenced FgTor and FgFKBP12 genes from this mutant. While no mutation was found in FgFKBP12, we discovered a single base-pair mutation (C to T at nucleotide 5597) in FgITOR resulting in a substitution of serine to leucine at codon 1866 (S1866L) (Fig. 3a). Intriguingly, this mutation is predicted to be located in the second alpha-helix of the Fkbp12-rapamycin binding (FRB) domain (Fig. S4b). Based on these observations, we hypothesized that FgTorS1866L is no longer recognized by or binding to the FgFkbp12-rapamycin complex. To test this, the physical interaction of FgFkbp12 with the FRB domain of FgTor (named FgFRB) was examined by yeast two-hybrid (Y2H) assays. Due to the fact that the WT yeast strain is sensitive to rapamycin, we generated a rapamycin-resistant yeast strain AH109R by UV radiation before this Y2H experiment. The pair of yeast two-hybrid plasmids was co-transformed into the rapamycin-resistant strain AH109R and, as shown in Fig. 3(b), FgFkbp12 was unable to interact with FgFRB without rapamycin treatment. By contrast, when the medium was supplemented with 1 l gm l⁻¹ rapamycin, FgFkbp12 interacted strongly with FgFRB. As expected, FgFkbp12 was unable to interact with the mutated FgFRBS1866L, regardless of rapamycin presence or absence. These results indicate that the point mutation (S1866L) prevents Fkbp12-rapamycin complex from binding to the FgFRB domain of FgTor.

FgTap42 interacts with FgSit4, FgPp2A and FgPpg1 in F. graminearum

In S. cerevisiae, Tor kinases execute their functions at least in part by regulating Tap42, a phosphatase 2A-associating protein (Di Como & Arndt, 1996). The F. graminearum genome has a single
TAP42 homolog (FGSG_09800, named FgTAP42). The predicted amino acid sequence of FgTap42 shares 29% similarity to S. cerevisiae Tap42. In order to investigate the function of FgTap42, we targeted FgTAP42 for gene deletion. Altogether, we obtained 65 transformants from four independent transformation experiments. However, none proved to be the FgTAP42 targeted gene deletion mutant, which led us to conclude that FgTAP42 may be an essential gene in F. graminearum. This conclusion is in agreement with a previous finding that Tap42 is an essential protein in S. cerevisiae (Di Como & Arndt, 1996).

In order to further determine the function of FgTap42, we assayed whether FgTAP42 could complement a temperature-sensitive S. cerevisiae tap42-11 mutant that can grow at 30°C but not at 37°C. The full-length FgTAP42 cDNA was cloned into pYES2 and transformed into the tap42-11 mutant. The growth defect of tap42-11 at 37°C was partially restored in the yeast transformant expressing FgTAP42 (Fig. 4a), indicating that the functions of Tap42 orthologs may be conserved in the budding yeast and filamentous fungi.

In S. cerevisiae, Tap42-phosphatase complexes associate with TORC1 and exist mainly on membrane structures (Yan et al., 2006). Comparisons in the subcellular localization of FgTap42 in the budding yeast and that in F. graminearum may provide some clues for understanding its biological functions. As shown in Fig. 5(a), FgTap42 was distributed mainly in cytoplasm in the S. cerevisiae tap42-11 strain. However, in F. graminearum, FgTap42 was localized in the cytoplasm in mycelia and near the nucleus in conidia (Fig. 5b,c), indicating that FgTap42 may perform distinct functions in mycelia and in conidia. These results are consistent with the observation that rapamycin is highly effective against hyphal growth (Fig. 1), but not against conidial germination of F. graminearum (data not shown).

Tap42 interacts with Tip41 (a Tap42-interacting protein) in S. cerevisiae (Jacinto et al., 2001). However, interaction between FgTap42 and FgTip41 (FGSG_06963) in F. graminearum was not observed in the Y2H assays (Fig. 6a). In S. cerevisiae, Tap42 also interacts with the catalytic subunit of type 2A protein phosphatases (PP2As), including Pph3, Pph21, Pph22 and the type 2A-like phosphatases, Sit4 and Ppg1 (Di Como & Arndt, 1996; Wang et al., 2003). The F. graminearum genome contains three genes, FGSG_09815, FGSG_01464 and FGSG_05281, named FgPP2A, FgSIT4 and FgPPG1, respectively, that encode putative orthologs of Pph21/Pph22/Pph3, Sit4 and Ppg1 (Tables S1, S3). Y2H assays showed that FgTap42 interacts with FgPP2A, FgSIT4 and FgPPG1 (Fig. 6a). The interaction of FgTap42 with FgSIT4 and FgPP2A was further confirmed by affinity capture and co-immunoprecipitation assays (Table 1, Fig. 6b). In addition, FgPPg1 also interacted with FgTip41 in Y2H and Co-IP assays (Fig. 6a,b). These results suggest that the interaction framework of FgTap42, FgTip41 and FgPPg1 in F. graminearum is different from that observed in S. cerevisiae.

FgPP2A, FgSIT4 and FgPPg1 could partially complement the yeast sit4 mutant

In order to characterize the functions of FgPP2A, FgSIT4 and FgPPG1, we tested whether these genes can complement the yeast
sir4 mutant (Hayashi et al., 2005). An expression vector pYES2 containing the full-length FgPP2A, FgSIT4 or FgPPG1 cDNA was transformed into the sir4 mutant, BY4741ΔSIT4. As a negative control, the mutant was also transformed with an empty pYES2 vector. As shown in Fig. 4(b), the growth defect of the yeast BY4741ΔSIT4 mutant at 37°C was partially restored by F. graminearum FgPP2A, FgSIT4 and FgPPG1. These results indicated that FgPP2A, FgSIT4 and FgPPG1 could function as the type 2A-like phosphatases in S. cerevisiae.

FgSit4 and FgPpg1 are important for hyphal development, virulence and sexual reproduction in F. graminearum

In order to determine the biological functions of FgPP2A, FgSIT4 and FgPPG1, we attempted to delete each gene in F. graminearum. For FgPP2A, we failed to obtain a null mutant after screening over 100 hygromycin-resistant transformants, indicating that it is likely an essential gene in F. graminearum. By contrast, the FgSIT4 deletion mutant (ΔFgSIT4) and FgPPG1 deletion mutant (ΔFgPPG1) grew remarkably more slowly than the WT strain on PDA (Fig. 7a). Nile Red staining showed that only few lipid droplets were observed in the hyphae of these mutants treated with 0.25 μg ml⁻¹ rapamycin (Fig. 2a), indicating that accumulation of lipid droplets in F. graminearum triggered by rapamycin is dependent on FgSit4 and FgPpg1.

Sexual reproduction is a critical aspect of the F. graminearum lifecycle (Trail, 2009; Min et al., 2012). Deletion of either FgSIT4 or FgPPG1 abolished the production of perithecia on carrot agar (Fig. 7b). In infection assays with flowering wheat heads, ΔFgSIT4 and ΔFgPPG1 caused scab symptoms only in the

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**Fig. 4** Complementation of the yeast mutants with Fusarium graminearum gene counterparts. (a) F. graminearum FgTAP42 partially complemented a yeast temperature-sensitive (ts) mutant tap42-11. The BY4741-derived ts mutant tap42-11 was transformed with pYES2 or pYES2-FgTAP42. Yeast cells containing BY4741, tap42-11+pYES2 or tap42-11+pYES2-FgTAP42 were spotted on yeast extract/peptone medium containing 2% galactose medium (YPG) and incubated at 30 or 37°C for 4 d. (b) FgPP2A, FgSIT4 and FgPPG1 partially complemented the growth defect of yeast SIT4 deletion mutant under 37°C. The yeast SIT4 mutant was complemented with FgPP2A, FgSIT4 or FgPPG1 cDNA to generate the strain BY4741ΔSIT4+pYES2-FgPP2A, BY4741ΔSIT4+pYES2-FgSIT4, or BY4741ΔSIT4+pYES2-FgPPG1, respectively. The wild-type (WT) strain BY4741 and SIT4 mutant BY4741ΔSIT4 transformed with empty pYES2 vector were used as controls. (c) FgTIP41 partially restored rapamycin sensitivity of the yeast TIP41 mutant. The yeast TIP41 mutant was complemented with FgTIP41 to generate the strain BY4741ΔTIP41+pYES2-FgTIP41. The WT strain BY4741 and TIP41 mutant BY4741ΔTIP41 transformed with empty pYES2 vector were used as controls. (d) FgAREA partially complements the Saccharomyces cerevisiae GLN3 mutant. The yeast GLN3 mutant was complemented with FgAREA cDNA to generate the strain BY4741ΔGLN3+pYES2-FgAREA. The WT strain BY4741 and GLN3 mutant BY4741ΔGLN3 transformed with empty pYES2 vector were used as controls. Serial dilutions of cell suspension of each strain were spotted on yeast peptone galactose medium (YPG) under different stresses as indicated in the figure.

**Fig. 5** Subcellular localization of FgTap42 in Saccharomyces cerevisiae and Fusarium graminearum. (a) Temperature-sensitive S. cerevisiae tap42-11 was complemented with GFP-tagged FgTap42. FgTap42-GFP was mainly observed in cytoplasm in S. cerevisiae. In F. graminearum, FgTap42-GFP was mainly localized to cytoplasm in hyphae (b) and to the nucleus in conidia (c). Nuclei in F. graminearum conidia were stained with 4′,6-diamidino-2-phenylindole (DAPI). DIC, differential interference contrast; bars, 10 μm.
inoculated spikelets (Fig. 8a). Under the same conditions, however, scab symptoms developed in > 90% spikelets when wheat heads were point-inoculated with the WT PH-1 or the complemented strains DFgSIT4-C and DFgPPG1-C (Fig. 8a).

FgSit4 and FgPpg1 show different roles in regulating conidiation and DON biosynthesis

Because DON is one of the important mycotoxins produced by F. graminearum, we assayed DON biosynthesis in the DFgSIT4 and DFgPPG1 mutants. After 20 d of incubation on sterilized wheat kernels, DFgPPG1 produced no detectable DON while the level of DON produced by DFgSIT4 was not significantly different from that of the WT strain (Fig. 8b). These results indicated that DFgSIT4 and DFgPPG1 play different roles in asexual development and secondary metabolism in F. graminearum.

When grown in MBB, DFgPPG1 produced relatively few conidia, whereas the DFgSIT4 produced similar numbers of conidia as the WT strain (Fig. 9a). Interestingly, conidia of DFgSIT4 and DFgPPG1 had fewer septa than those of the WT strain (Fig. 9b), indicating that FgSit4 and FgPpg1 may be involved in regulating septum formation.

Involvement of FgSit4 and FgPpg1 in cell wall integrity

In order to further explore the specific function of FgSit4 and FgPpg1 in F. graminearum, we prepared serial dilutions of spores of each mutant and placed them under a variety of stress conditions. ΔFgSIT4 and ΔFgPPG1 exhibited dramatically increased

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Table 1 Putative FgTap42, FgSit4 and FgPpg1 interacting proteins identified by affinity capture assays in Fusarium graminearum

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Fig. 6 Yeast two-hybrid and co-immunoprecipitation (Co-IP) analyses of interactions among FgTap42, FgTip41 and the protein phosphatases FgPp2A, FgSit4 and FgPpg1 from Fusarium graminearum. (a) Serial dilutions of yeast cells (cells ml⁻¹) transferred with the bait and prey constructs indicated in the figure were assayed for growth on yeast minimal synthetic defined base (SD) depleted of leucine, tryptophan, and histidine. The pair of plasmids pGBK7-53 and pGAD7T7 was used as a positive control. The pair of plasmids pGBK7-Lam and pGAD7T7 was used as negative control. The same set of yeast transformants were also assayed for β-galactosidase activity. (b) Co-IP assays. Immunoblots of total proteins extracted from F. graminearum transformants co-expressing the GFP and FLAG fusion constructs as indicated and proteins eluted from anti-FLAG agarose were detected with monoclonal anti-FLAG and monoclonal anti-GFP antibodies, respectively. Flow-through solution and total proteins isolated from the wild-type progenitor PH-1 served as controls.
sensitivity to the cell wall damaging agents Congo red and calcofluor white (Fig. 10), but not to osmotic stress mediated by NaCl, KCl and sorbitol, and oxidative stress mediated by H2O2 (data not shown). Western blot analysis showed that the phosphorylation level of FgMgv1 in the deletion mutant (Fig. 11b). In addition, the deletion of FgTIP41 on wheat kernels was decreased > 60% in comparison with the WT strain (Fig. 8b). These results indicated that FgTIP41 is involved in regulation of DON biosynthesis, subsequently affecting virulence in F. graminearum.

FgAreA is associated with conidiation, mycotoxin production and pathogenicity

In S. cerevisiae, the transcriptional activators Gln3 and Gat1 (Nil1) are phosphorylated in a TOR-dependent manner under N-sufficient conditions (Rohde & Cardenas, 2004). In the filamentous fungus Aspergillus nidulans, the major regulator of N metabolism, the GATA factor AreA (Kudla et al., 1990), is an ortholog of Gln3 and Gat1. The F. graminearum genome contains one AREA ortholog (FGSG_08634) named FgAREA. When expressed in yeast, FgAREA could partially restore tolerance of the Gln3 mutant to rapamycin and paraquat (Fig. 4d). Consistent with an earlier report on systematic characterization of AreA in F. graminearum (Min et al., 2012), FgAREA was impaired in growth on a medium containing nitrate as the sole N source, and showed severely reduced virulence and DON production on wheat heads (Fig. S5).

Discussion

The benzimidazole and triazole fungicides, which have been used widely for the control of FHB, normally provide only c. 50%
result in lipid vesicle aggregation (Zhang et al., 2000). Based on these results, we presumed that rapamycin may have similar effects on lipid metabolism in filamentous fungi and in insects.

In S. cerevisiae, rapamycin exerts antifungal activity via its interaction with the prolyl isomerase Fkbp12 to form a binary complex, which binds to the conserved FRB domain of Tor kinases. Fkbp12 catalyzes cis-trans peptidyl-prolyl isomerization, a rate-limiting step in protein folding (Hur & Bruice, 2002). In our current study, we found that the FgFKBP12 deletion mutant of F. graminearum was resistant to rapamycin and FK506 (Fig. S3). Resistance of FKB12 deletion mutants to rapamycin has been documented in several other fungi, including A. nidulans, B. cinerea, Candida albicans, Cryptococcus neoformans, Fusarium fujikuroi and Mucor circinelloides (Cruz et al., 1999, 2001; Fitzgibbon et al., 2005; Teichert et al., 2006; Melendez et al., 2009; Bastidas et al., 2012). In addition, rapamycin affects sexual development rather than vegetative growth, and this development is mediated by Fkbp12 via a mechanism independent of the TOR pathway, as shown in fission yeast (Weisman et al., 2001). In addition, the deletion of FKB12 (named BcPICS) in B. cinerea strain T4 led to reduced virulence on Arabidopsis thaliana. By contrast, deletion of BcFPKP12 did not impair virulence in B. cinerea strain B05.10 on tomato and grape fruit, and even led to faster colonization of apple sections and cucumber cotyledons (Melendez et al., 2009). These results strongly indicate that roles of Fkb12 orthologs vary considerably in different fungi, likely in a species-specific or even strain-specific manner.

In S. cerevisiae, the Tor downstream effector Tap42 is an essential protein (Di Como & Arndt, 1996). Similarly, we were unable to obtain a F. graminearum FgTap42 null mutant. Complementation of a temperature-sensitive S. cerevisiae tap42-11 mutant with FgTap42 further indicates that Tap42 protein has conserved functions in yeast and filamentous fungi. In S. cerevisiae, the interaction of Tap42 with PP2A is regulated development rather than vegetative growth, and this development is mediated by Fkbp12 via a mechanism independent of the TOR pathway, as shown in fission yeast (Weisman et al., 2001). In addition, the deletion of FKB12 (named BcPICS) in B. cinerea strain T4 led to reduced virulence on Arabidopsis thaliana. By contrast, deletion of BcFPKP12 did not impair virulence in B. cinerea strain B05.10 on tomato and grape fruit, and even led to faster colonization of apple sections and cucumber cotyledons (Melendez et al., 2009). These results strongly indicate that roles of Fkb12 orthologs vary considerably in different fungi, likely in a species-specific or even strain-specific manner.

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and PP2A-like proteins (e.g. FgPp2A, FgSit4 and FgPpg1), and all of these genes partially complemented the yeast sit4 mutant (Fig. 4b). Unlike S. cerevisiae, where only Sit4 plays a critical role in cell growth, all three FgSit4-interacting phosphatases are important for fungal growth in F. graminearum; the deletion of FgPP2A is predicted to be lethal, and the deletion of either FgSIT4 or FgPpg1 led to severely reduced mycelial growth.

Comparative analysis showed that FgSit4 and FgPpg1 are homologous to S. cerevisiae Sit4 and Ppg1, respectively, whereas FgPp2A is homologous to Pph3, Pph21 and Pph22 (Table S3). Similar to S. cerevisiae Sit4, FgSit4 also plays important roles in regulation of various cellular processes, including mycelial growth, virulence and sexual development in F. graminearum. In S. cerevisiae, while deletion of PPH21 or PPH22 did not result in notable phenotypic deficiencies, double mutation eliminated 80–90% of total PP2A activity in the cell and led to severely crippled growth (Sneddon et al., 1990). The residual PP2A activity in the absence of Pph21 and Pph22 is believed to be contributed by Pph3, because the deletion of PPH3 is lethal in the Pph21/Pph22 double mutant (Sneddon et al., 1990). Because F. graminearum contains a single FgPP2A, which is homologous to PPH3, PPH21, and PPH22 (Table S3), it was not surprising to discover that the deletion of FgPP2A in F. graminearum is lethal.
In *S. cerevisiae*, deletion of *PPG1* has no obvious effects on cell growth (Jiang, 2006). However, deletion of its ortholog Fg*PPG1* in *F. graminearum* resulted in various defects, including reduced mycelial growth and impaired asexual and sexual development. Subsequently, we hypothesized that the important functions of Fg*Ppg1* in *F. graminearum* are mediated by its interaction with Fg*Tip41*. Tip41 was identified as a binding partner of Tap42 in the budding yeast, and has been suggested as a regulator of the rapamycin-sensitive signaling pathway by competing for Tap42 against Sir4 (Jacinto et al., 2001). Interestingly, we found that Fg*Tip41* interacts with Fg*Ppg1* rather than Fg*Tap42*, which may explain why Fg*Ppg1* was not co-purified with Fg*Tap42* in our affinity capture assays (Table 1). In addition, we also observed the interaction between Fg*Ppg1* and Fg*Tap42* in the Y2H assay, indicating that Fg*Tap42*, Fg*Ppg1* and Fg*Tip41* may form a heterotrimer in *F. graminearum*. To our knowledge, this is the first report on the existence of this heterotrimer (Fg*Tip41*: Fg*Ppg1*:Fg*Tap42*) in a fungal species. Our finding is in contrast with the model proposed in *S. cerevisiae*, but in agreement with recently published study on human proteins. The mammalian ortholog of Tip41 (TIPRL) does not interact with the mammalian Tap42 ortholog (α4), but TIPRL and α4 can bind PP2A simultaneously, forming a stable ternary complex, which regulates the activity of PP2A (Murata et al., 1997).

Fg*Sit4* and Fg*Ppg1* appear to be involved in several functions including vegetative development and virulence in *F. graminearum*. In this study, we found for the first time that Fg*Sit4* and Fg*Ppg1* positively regulate phosphorylation of Fg*Mgv1* via interacting with a negative regulator Fg*Msg5*, which is a negative regulator of the CWI pathway (Flandez et al., 2004). Because the CWI pathway is known to carry out various cellular functions including regulating virulence, mycelial growth, sexual and asexual development, and secondary metabolism in *F. graminearum* (Hou et al., 2002), Fg*Sit4* and Fg*Ppg1* may be involved in regulating various cellular processes partially via the CWI pathway in *F. graminearum*. In addition, SAGE analyses of gene expression profiling revealed that several transcription factor genes (FGSG_05304, FGSG_06542 and FGSG_01877) were highly upregulated in the Fg*PPG1* deletion mutant (data not shown). Similar to the Fg*PPG1* mutant, deletion mutants of these transcription factor genes exhibit reduced mycelial growth rate, decreased DON production, less conidiation or impaired virulence on wheat head (Son et al., 2011), indicating that Fg*Ppg1* may regulate a variety of regulatory pathways via different transcription factors (Fig. 12).

Inhibition of TOR by rapamycin causes a nutrient stress response in *S. cerevisiae*. Both N starvation and rapamycin treatment resulted in rapid dephosphorylation of Gln3 by Sir4 (Beck & Hall, 1999). Diphosphorylated Gln3 is imported into the nucleus, and activates genes involved in the assimilation of alternative N sources (Beck & Hall, 1999; Cooper, 2002). The functions of Tor in N metabolism remain controversial in the filamentous fungus *A. nidulans*. Tor was proposed to have only a minor role in N metabolism in *A. nidulans* because mutations in the TOR pathway genes did not have notable effects on the N utilization phenotype (Fitzgibbon et al., 2005). However, in *Fusarium oxysporum*, rapamycin treatment increases transcript levels of genes involved in N catabolism, such as *NIT1*, supporting a role of TOR in N catabolite repression (Lopez-Berges et al., 2010). This finding is in agreement with a previous observation that rapamycin activates expression of N metabolism–related genes encoding ammonium permease and uricase in *Fusarium fujikuroi* (Teichert et al., 2006). Similar to these findings, we found that rapamycin treatment led to increased expressions of several genes involved in N metabolism in *F. graminearum*, such as genes encoding 2-nitropropane dioxygenase and NAD-specific glutamate dehydrogenase (data not shown). Furthermore, the *F. graminearum* ortholog of Gln3, Fg*AreA*, could fully complement the budding yeast *GLN3* deletion mutant (Fig. 4d). In addition, ΔFG ARE A and ΔFg*Ppg1* showed similar defects in conidiation, DON production and pathogenicity. Collectively, we propose that Fg*AreA* is one of the Fg*Ppg1* downstream components in the TOR signaling pathway in *F. graminearum* (Fig. 12).

In conclusion, we found that the TOR pathway plays critical roles in regulating vegetative differentiation and virulence in *F. graminearum*, which advances our understanding of the pathogenesis of plant pathogenic fungi.

**Acknowledgements**

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References


Cooper TG. 2002. Transmitting the signal of excess nitrogen in Saccharomyces cerevisiae from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiology Reviews 26: 223–238.


Murata K, Wu J, Brautigan DL. 1997. B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein
phosphatase 2A. *Proceedings of the National Academy of Sciences, USA* 94: 10 624–10 629.


**Supporting Information**

Additional supporting information may be found in the online version of this article.

Fig. S1 Southern blot hybridization analyses of ΔFgFKBP12, ΔFgFKBP20 and ΔFgFKBP54 of *Fusarium graminearum* on potato dextrose agar (PDA) plates and their sensitivity to rapamycin and FK506.

Fig. S2 The colony morphology of the mutants ΔFgFKBP12, ΔFgFKBP20 and ΔFgFKBP54 of *Fusarium graminearum* showed resistance to rapamycin and FK506.

Fig. S3 The *FgFKBP12* deletion mutant (ΔFgFKBP12) of *Fusarium graminearum* reduced virulence of *FgTor* kinase of *Fusarium graminearum*.

Fig. S4 A rapamycin-resistant (RR) mutant generated by UV irradiation harbors a point mutant at the codon 1866 in the Fkb12-rapamycin binding (FRB) domain of FgTor kinase of *Fusarium graminearum*.

Fig. S5 Nitrogen (N) utilization, virulence and deoxynivalenol (DON) production of ΔFgAREA of *Fusarium graminearum*.

**Table S1** Summary of the components of the target of rapamycin (TOR) pathway in *Fusarium graminearum* and their orthologs in *Saccharomyces cerevisiae*.

**Table S2** A total of 138 oligonucleotide primers were used in this study.

**Table S3** Genes encoding catalytic subunits of type 2A phosphatase and type 2A-like phosphatase in *Fusarium graminearum*.

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