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ORIGINAL ARTICLE

Constitutive activation of TORC1 signalling attenuates virulence in the cross-kingdom fungal pathogen *Fusarium oxysporum*

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Abstract

The filamentous fungus *Fusarium oxysporum* causes vascular wilt disease in a wide range of plant species and opportunistic infections in humans. Previous work suggested that invasive growth in this pathogen is controlled by environmental cues such as pH and nutrient status. Here we investigated the role of Target Of Rapamycin Complex 1 (TORC1), a global regulator of eukaryotic cell growth and development. Inactivation of the negative regulator Tuberous Sclerosis Complex 2 (Tsc2), but not constitutive activation of the positive regulator Gtr1, in *F. oxysporum* resulted in inappropriate activation of TORC1 signalling under nutrient-limiting conditions. The *tsc2*Δ mutants showed reduced colony growth on minimal medium with different nitrogen sources and increased sensitivity to cell wall or high temperature stress. Furthermore, these mutants were impaired in invasive hyphal growth across cellophane membranes and exhibited a marked decrease in virulence, both on tomato plants and on the invertebrate animal host *Galleria mellonella*. Importantly, invasive hyphal growth in *tsc2*Δ strains was rescued by rapamycin-mediated inhibition of TORC1. Collectively, these results reveal a key role of TORC1 signalling in the development and pathogenicity of *F. oxysporum* and suggest new potential targets for controlling fungal infections.

KEYWORDS
fungal pathogenicity, *Fusarium oxysporum*, Gtr1, TORC1, Tsc2

1 | INTRODUCTION

Soilborne fungal plant pathogens are ubiquitous and highly persistent, causing massive losses in field and greenhouse crops. Fungicide application, resistance breeding, and crop rotation are agricultural practices that have proven insufficient to prevent root diseases (Haas & Defago, 2005). The soil-inhabiting ascomycete *Fusarium oxysporum* causes vascular wilt in more than 150 different plant species and ranks among the most important phytopathogens (Dean et al., 2012). Furthermore, this fungus can produce opportunistic infections in humans ranging from superficial or locally invasive to disseminated, depending on the immune status of the patient (Nucci & Anaissie, 2007). Strikingly, a single *F. oxysporum* f. sp. *lycopersici* isolate can infect and kill both tomato plants and immunosuppressed mice (Ortoneda et al., 2004), making this an excellent model to study the genetic basis of cross-kingdom pathogenesis in fungi.

Previous studies established that plant infection by *F. oxysporum* is regulated by a network of conserved signalling pathways that sense and transduce environmental cues such as nutrients, ambient pH, and host compounds (Caracuel et al., 2003; Di Pietro et al., 2001; López-Berges et al., 2010a; Masachis et al., 2016; Turra et al., 2015). One of the key pathogenicity mechanisms, invasive hyphal growth, was found to be dependent on nutrient status and promoted by rapamycin, a specific inhibitor of the protein kinase Target Of Rapamycin (TOR) (López-Berges et al., 2010a). TOR is broadly conserved in eukaryotes and controls nutrient signalling (Wang & Proud, 2009) and cell growth from yeast to mammals (Gonzalez & Hall, 2017; Loewith & Hall, 2011; Schmelzle & Hall, 2000). TOR interacts with other proteins to form two structurally and functionally distinct complexes, TOR complexes 1 (TORC1) and 2 (TORC2) (Gonzalez & Hall, 2017; Loewith & Hall, 2011; Schmelzle & Hall, 2000). The rapamycin-sensitive TORC1 promotes cell growth in response to nutrients, growth factors, and cellular energy by activating anabolic processes such as ribosome biogenesis and protein synthesis, and preventing catabolic processes such as autophagy and ubiquitin-mediated proteolysis (Saxton & Sabatini, 2017), while TORC2 is insensitive to rapamycin and controls actin cytoskeleton organization (Wullschleger et al., 2006). Activity of TORC1 is positively and negatively regulated, respectively, by the small 'Ras homolog enriched in brain' GTPase Rheb (Long et al., 2005) and its GTPase-activating protein (GAP) Tsc2 (Wullschleger et al., 2006). A second upstream component is the vacuolar membrane associated 'escape from rapamycin-induced growth arrest' EGO protein complex (Binda et al., 2009; Loewith & Hall, 2011), with the small GTPases Gtr1/Gtr2 acting as TORC1 activators (Binda et al., 2009).

Currently, the role of TORC1 in fungal pathogenicity on plants is controversial. Genetic or pharmacological inhibition of TORC1 does affect virulence in different phytopathogens such as *Botrytis cinerea*, *Verticillium dahliae*, *Fusarium graminearum*, and *F. oxysporum*, but this could be explained by a severe reduction of mycelial growth (Li et al., 2019, 2021; Xiong et al., 2019; Yu et al., 2014). On the contrary, studies in the rice blast fungus *Magnaporthe oryzae* point towards an inhibitory function of TORC1 in appressorium formation and other virulence-related processes such as autophagy (Marroquin-Guzman & Wilson, 2015; Sun et al., 2019). In line with this, we previously found that pharmacological inhibition of TORC1 by rapamycin promotes invasive growth in *F. oxysporum*, suggesting that TORC1 negatively regulates pathogenicity functions (López-Berges et al., 2010a).

Here we investigated the effect of constitutive TORC1 activation through targeted deletion of *tsc2* (*tsc2*Δ) and/or expression of a constitutively active $gtr1^{Q86L}$ allele ($gtr1^{GTP}$), on the development and pathogenesis of *F. oxysporum*. We found that inappropriate activation of TORC1 in *tsc2*Δ strains leads to impaired colony growth under nutrient-limiting conditions, increased sensitivity to stresses, impaired invasive growth, and reduced virulence on plant and animal hosts, highlighting the crucial role of TORC1 regulation during fungal development and pathogenicity.

2 | RESULTS

2.1 | Establishment of TORC1 readouts in *F. oxysporum*

To measure TORC1 activity in *F. oxysporum*, we first tested the feasibility of two different readouts used previously in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*: transcriptional activation of ribosomal genes (Hannan et al., 2003; Martin et al., 2004) and phosphorylation of the ribosomal protein S6 (Rps6) (Gonzalez et al., 2015; Sato et al., 2009). For the first readout we monitored transcript levels of the *F. oxysporum* ribosomal genes *FOXG_01615*, *FOXG_07725*, and *FOXG_09465*, encoding 50S ribosomal protein L4e, 40S ribosomal protein S5, and 40S ribosomal protein S3, respectively, while for the second readout we determined the phosphorylation status of *F. oxysporum* Rps6 using a phospho-Akt antibody (see experimental procedures).

In a short-term adaptation experiment, *F. oxysporum* germlings were either transferred from minimal medium lacking a nitrogen source (−N) to minimal medium with glutamine (Gln) or maintained in −N conditions. A shift from −N to Gln resulted in rapid up-regulation of the three ribosomal genes and a marked increase in Rps6 phosphorylation, both indicative of TORC1 activation (Figure 1a). By contrast, the inverse shift from Gln to −N caused a decrease in both readouts that was further exacerbated in the presence of 100 ng/ml rapamycin (−N + R) (Figure 1b). Importantly, the same effect of rapamycin treatment was observed even when the fungus was maintained in Gln, confirming the inhibitory effect of this drug on TORC1 activity (Figure 1c). We conclude that levels of ribosomal gene transcripts and of Rps6 phosphorylation can be used to reliably monitor TORC1 activity in *F. oxysporum*.

2.2 | Targeted deletion of *tsc2*, but not expression of a constitutively active $gtr1^{Q86L}$ allele ($gtr1^{GTP}$), activates TORC1 signalling in *F. oxysporum*

We attempted two different approaches for constitutive TORC1 activation in *F. oxysporum*: (1) deletion of *tsc2* (*tsc2*Δ) and (2) expression of a constitutively active $gtr1^{Q86L}$ allele ($gtr1^{GTP}$). A BlastP search in FungiDB (Basenko et al., 2018) using Tsc2 from *S. pombe* (SPAC630.13c) as bait detected a single *F. oxysporum* Tsc2 orthologue (FOXG_01632). ClustalW alignment (Thompson et al., 1994) of the GTPase-activating domain of Tsc2 from evolutionarily distant organisms showed a high degree of conservation (Figure S1). Isogenic *tsc2*Δ mutants of *F. oxysporum* were obtained by replacing the entire *FOXG_01632* coding sequence with the *hygromycin B* resistance gene (Figure S2). When the *tsc2*Δ mutant was submitted to a shift from Gln to −N, both ribosomal gene transcript and Rps6 phosphorylation levels remained largely stable, in contrast to the wild-type strain, suggesting that activation of TORC1 in the *tsc2*Δ mutant is independent of nutrient status (Figure 2a). As expected, rapamycin-mediated TORC1 inhibition still caused a decrease of

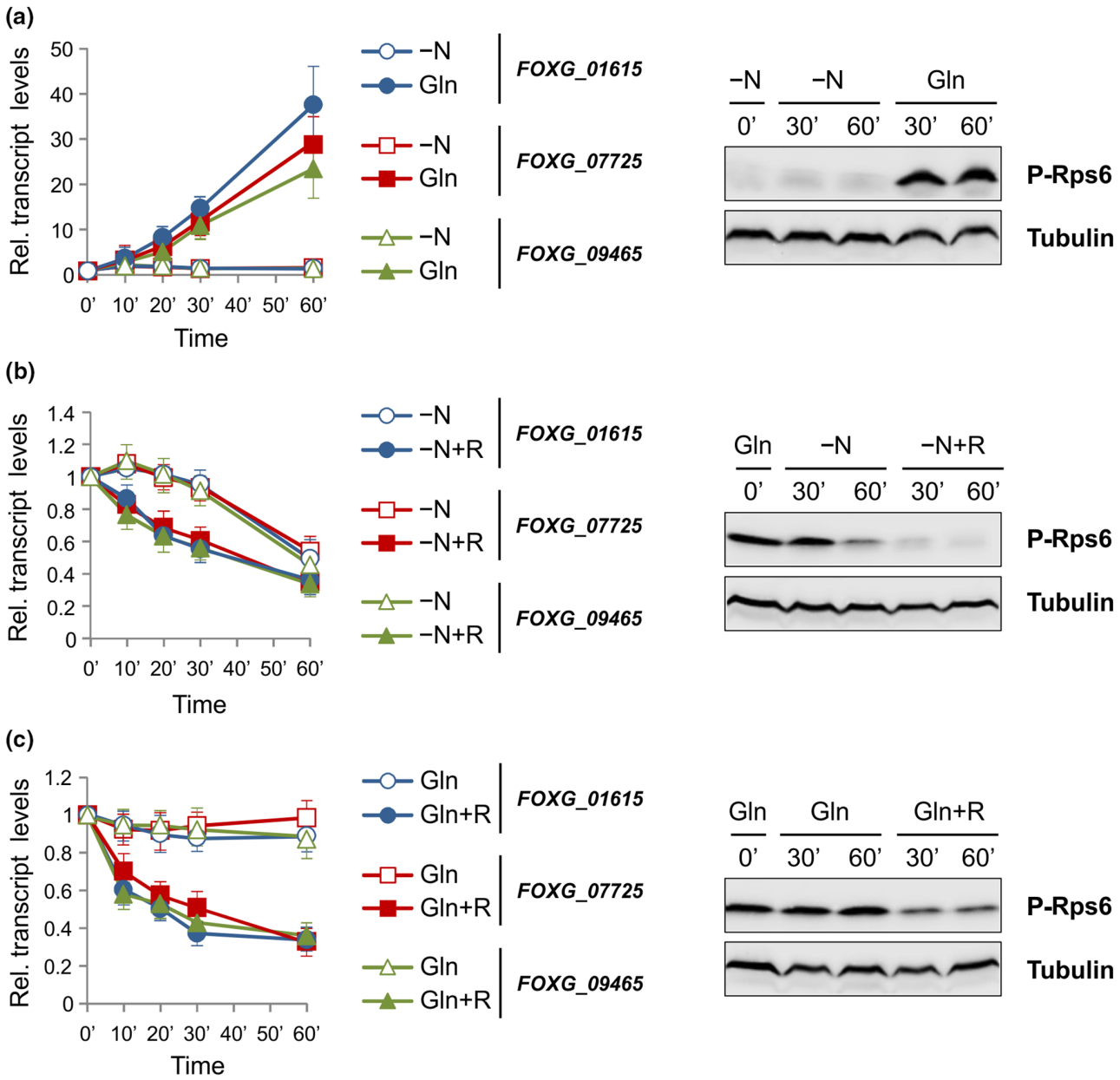

FIGURE 1 Establishment of TORC1 readouts in *Fusarium oxysporum*. (a–c) The wild-type strain was germinated for 16 h in potato dextrose broth (PDB) and then transferred to (a) nitrogen-free minimal medium (−N) for 60 min (time point 0′) before shifting either to −N (empty symbols) or glutamine minimal medium (Gln) (solid symbols) for an additional 60 min, (b) Gln for 60 min (time point 0′) before shifting either to −N (empty symbols) or −N + 100 ng/ml rapamycin (−N + R) (solid symbols) for an additional 60 min, or (c) Gln for 60 min (time point 0′) before shifting to Gln (empty symbols) or Gln + 100 ng/ml rapamycin (Gln + R) (solid symbols) for an additional 60 min. Left panels: Transcript levels of the indicated ribosomal genes were measured at the indicated time points by reverse transcription-quantitative PCR, normalized to the *act1* gene, and expressed relative to those obtained at time 0′. Bars represent standard deviations calculated from three independent experiments with three technical replicates each. Right panels: Representative western blots showing phosphorylation of the ribosomal protein S6 (Rps6) at the indicated time points. α-tubulin was used as loading control. Blots were performed with three biological replicates with similar results.

TORC1 readouts in *tsc2*Δ, consistent with pathway hyperactivation occurring upstream of TORC1 (Figure 2a). Importantly, reintroduction of the wild-type *tsc2* allele into *tsc2*Δ, yielding the complemented strain *tsc2*Δ^{C} (Figure S2), fully restored wild-type values for the TORC1 readouts on a shift from Gln to −N (Figure 2b).

In a second approach, we generated a strain in which Gtr1 was locked in a constitutively active (GTP-bound) form (*gtr1*^{GTP}) that was previously shown to activate TORC1 (Binda et al., 2009; Efeyan et al., 2013). A BlastP search using Gtr1 from *S. cerevisiae* (SGD:S000004590) as bait detected a single *F. oxysporum* Gtr1 orthologue (FOXG_07552). ClustalW alignment (Thompson et al., 1994) of the N-terminal region of Gtr1 from different organisms identified glutamine 86 (Q86) of *F. oxysporum* Gtr1 as the conserved residue to be replaced by leucine (L) to generate a

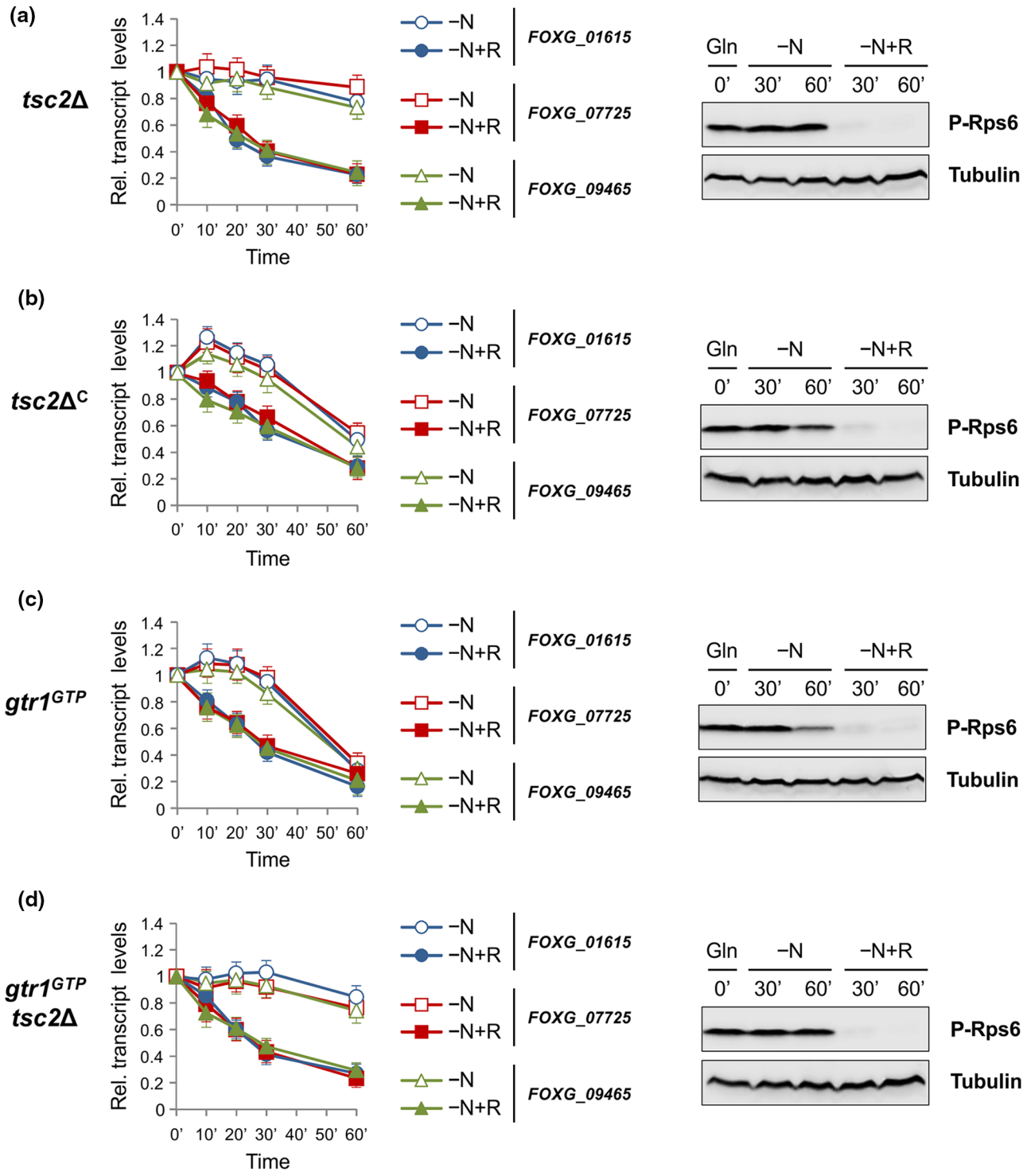

FIGURE 2 Targeted deletion of *tsc2* leads to nitrogen status-independent TORC1 activation. (a–d) Samples were obtained from the indicated strains as described in Figure 1b. Left panels: Transcript levels of the indicated ribosomal genes were measured at the indicated time points by reverse transcription-quantitative PCR, normalized to the *act1* gene, and expressed relative to those obtained at time 0′. Bars represent standard deviations calculated from three independent experiments with three technical replicates each. Right panels: Representative western blots showing phosphorylation of Rps6 at the indicated time points. α-tubulin was used as loading control. Blots were performed with three biological replicates with similar results.

GTP-locked Gtr1 variant (Efeyan et al., 2013; Gao & Kaiser, 2006; Nakashima et al., 1999) (Figure S3). The *gtr1*^{Q86L} allele was generated by site-directed mutagenesis and introduced into the wild-type strain by cotransformation with the *phleomycin B* resistance gene (Figure S4). Analysis of several phleomycin-resistant cotransformants by PCR amplification and sequencing of the relevant

genomic region identified a transformant carrying a discrete thymine (T) peak in position 257, indicating homologous replacement of the wild-type *gtr1* allele with $gtr1^{Q86L}$ in this strain (Figure S4c). In addition, we also obtained strains carrying both mutations by deleting the *tsc2* gene in the $gtr1^{GTP}$ background (Figure S5). We noted that the TORC1 readouts in the $gtr1^{GTP}$ mutant were very similar to those of the wild-type strain on a shift from Gln to −N, whereas those in the $gtr1^{GTP}$ *tsc2*Δ double mutant were comparable to the *tsc2*Δ single mutant (Figure 2c,d). We thus conclude that inactivation of Tsc2 in *F. oxysporum* leads to constitutive activation of TORC1 while expression of $gtr1^{GTP}$ has no detectable effect on TORC1 activity under the conditions tested.

2.3 | Constitutive activation of TORC1 affects *F. oxysporum* growth, development, and stress response

Growth of the different fungal strains was analysed in solid and liquid media. On plates, colony growth of the *tsc2*Δ mutant was

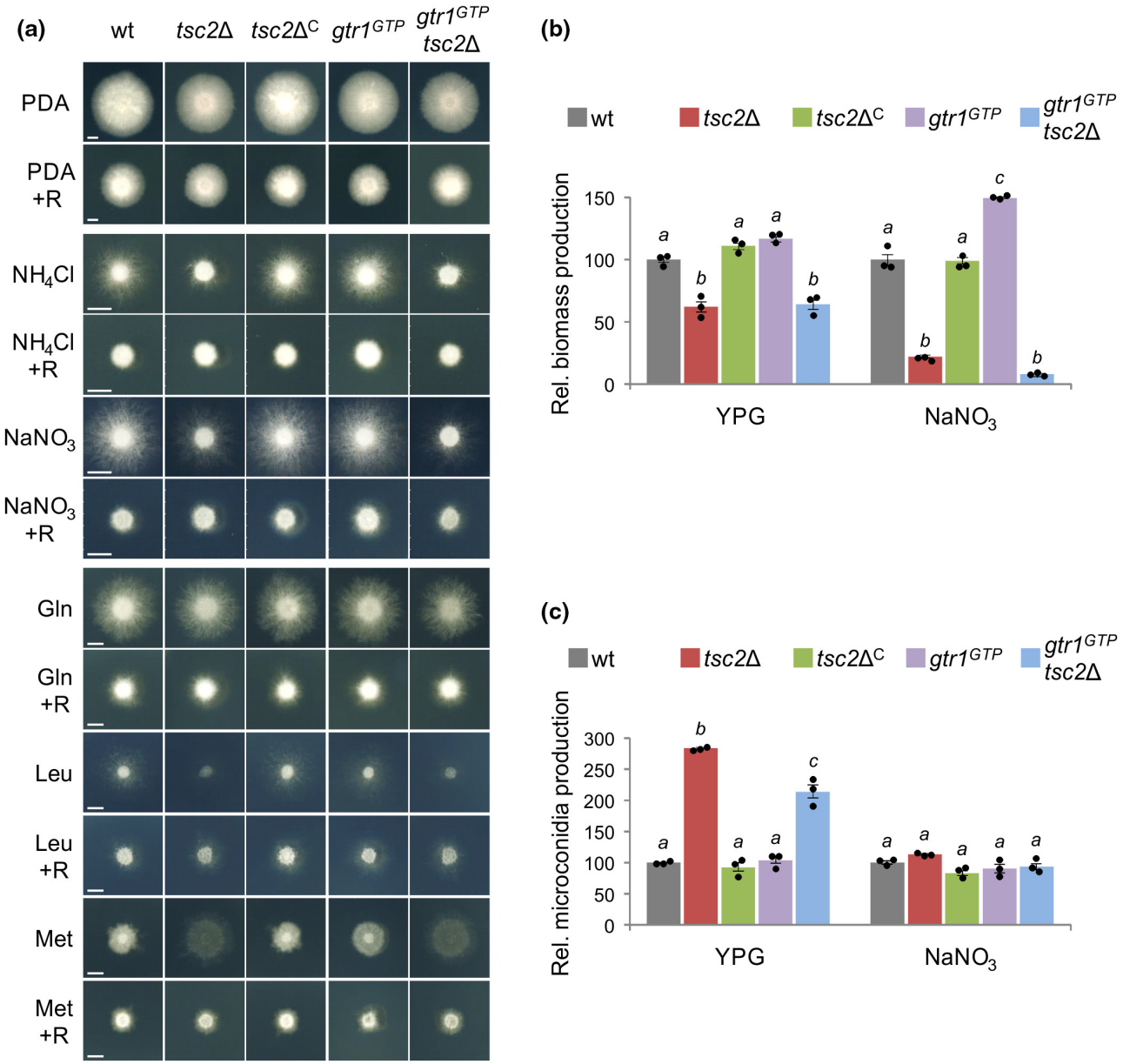

FIGURE 3 Constitutive activation of TORC1 affects growth and development of *Fusarium oxysporum*. (a) The indicated strains were spot-inoculated on potato dextrose agar (PDA) or on minimal medium (MM) supplemented with the indicated inorganic or organic nitrogen source, with or without 2 ng/ml rapamycin (R). Cultures were grown at 28°C for 3–4 days and imaged. Scale bars = 5 mm. (b, c) Biomass (dry weight) (b) and microconidia production (c) of the indicated strains after 48 h of growth in liquid yeast extract peptone glucose or in MM supplemented with $NaNO_3$ shaking and static cultures, respectively. Values are represented relative to those of the wild-type strain. Values with the same letter are not significantly different ($p < 0.05$) according to unpaired *t* test. Bars represent standard deviations calculated from three independent experiments with two replicates each.

generally reduced compared to that of the wild-type and the *tsc2*Δ^{C} strains, both in terms of diameter and hyphal density, but this effect was much more pronounced on minimal medium (Figure 3a). Importantly, the addition of rapamycin reduced colony growth to a similar extent in all strains tested. In liquid culture, biomass production of the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants was also significantly reduced compared to the wild-type strain, both in complete and minimal medium, but the effect was again more pronounced in the latter condition (Figure 3b). Moreover, microconidia production of the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants was significantly increased in complete, but not in minimal liquid medium (Figure 3c).

In contrast to the *tsc2*Δ mutant, growth of the *gtr1*^{GTP} mutant was largely similar to that of the wild-type strain under most conditions tested, except for a slight reduction on potato dextrose agar (PDA) plates and an increase in biomass in liquid minimal medium (Figure 3a,b).

We next tested growth of the strains in the presence of different stresses and found that the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants displayed increased sensitivity to the cell wall-targeting compound calcofluor white (CFW) and to high temperature stress (37°C) (Figure 4a,b). By contrast, no differences were detected in the presence of hyperosmotic (1 M NaCl, 1 M sorbitol) or oxidative (10 μg/ml menadione, 0.01% H_2O_2) stress conditions (not shown).

To further investigate the integrity of the cell wall during conidial germination, fluorescence microscopy analysis was performed using the chitin-binding dye CFW. Two hours after inoculation in potato dextrose broth (PDB), the microconidia of the wild-type, *tsc2*Δ^{C}, and *gtr1*^{GTP} strains showed a largely uniform fluorescence signal along the cell wall, whereas the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants showed a highly irregular distribution of fluorescence with a lower overall level, but strongly fluorescent punctae that were still detectable after 6–8 h (Figure 4c,d). Moreover, the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants exhibited a marked delay in germination compared to the wild type. Importantly, addition of rapamycin to the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants restored uniform distribution of the CFW fluorescence signal and germination to wild-type levels (Figure 4d). Taken together, these results indicate that constitutive activation of the TORC1 signalling pathway through *tsc2* deletion negatively affects *F. oxysporum* growth, development, and response to different stress conditions.

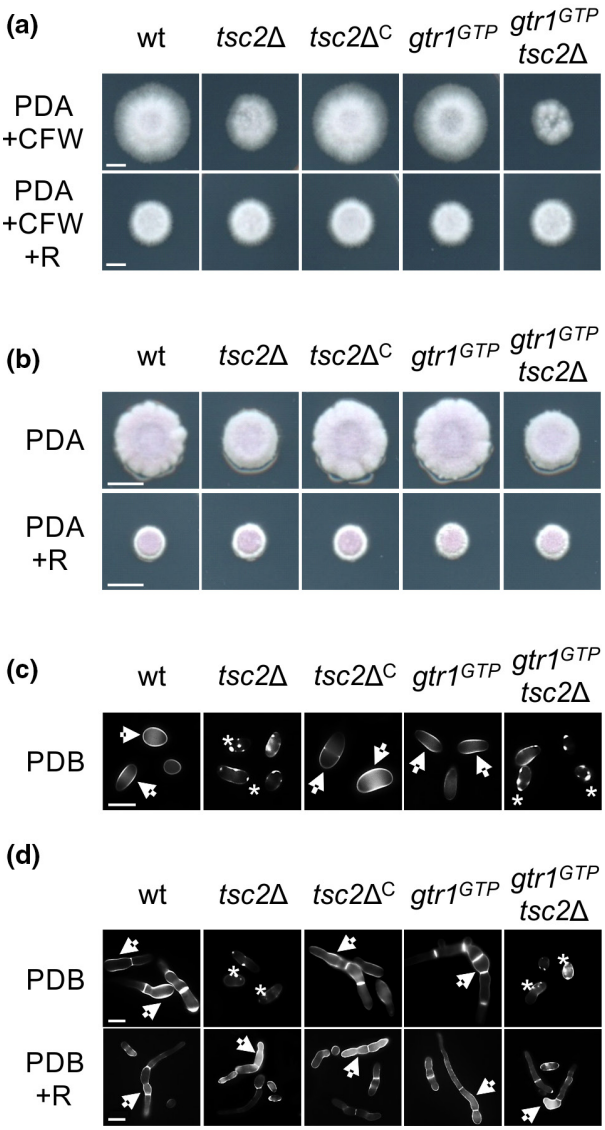

FIGURE 4 Constitutive activation of TORC1 increases sensitivity to cell wall and high temperature stress. (a, b) The indicated strains were spot-inoculated on potato dextrose agar (PDA) supplemented with 40 μg/ml calcofluor white (CFW) with or without 2 ng/ml rapamycin (R) and grown at 28°C for 3–4 days (a) or on PDA with or without 2 ng/ml rapamycin (R) and grown at 37°C for 4 days (b). Scale bars = 5 mm. (c, d) Microconidia of the indicated strains were germinated in potato dextrose broth with and without 2 ng/ml rapamycin for 2 h (c) or 6–8 h (d) and stained with 6 mg/ml CFW. Arrows highlight the uniform fluorescence signal along the cell wall while asterisks indicate irregular fluorescent punctae. Fluorescence microscopy images were taken at 100× magnification. Scale bars = 5 μm.

2.4 | TORC1 activation blocks invasive hyphal growth of *F. oxysporum*

Previous work established that fungal plant pathogenicity requires the correct activation of multiple virulence functions, including invasive hyphal growth, a process that is experimentally defined by the ability to penetrate across a cellophane membrane (López-Berges et al., 2010a, 2010b; Prados Rosales & Di Pietro, 2008). Here we found that, in contrast to the wild-type strain, the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants failed to penetrate cellophane membranes (Figure 5a). Importantly, invasive growth of these mutants was fully restored by addition of rapamycin (Figure 5b). In line with these findings, the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants caused less tissue maceration than the wild type when inoculated on apple slices, indicative of reduced invasive growth on living fruit tissue (Figure 5c) (López-Berges et al., 2009). Moreover, vegetative hyphal fusion, another virulence-related function that can be visualized by the formation of macroscopically visible hyphal aggregates (López-Berges et al., 2010a, 2010b; Prados Rosales & Di Pietro, 2008), was also impaired in *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ, and to a lesser extent in

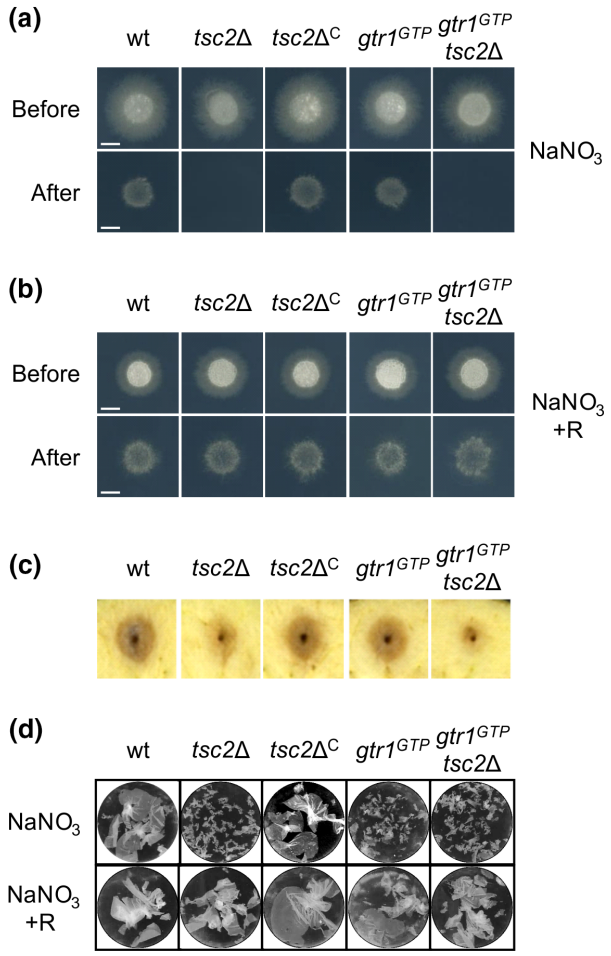

FIGURE 5 TORC1 negatively regulates virulence-related functions. (a, b) Cellophane penetration was determined on minimal medium (MM) plates containing 50 mM $NaNO_3$ without (a) or with (b) 2 ng/ml rapamycin (R). The indicated fungal strains were spot-inoculated and grown 4 days at 28°C on top of cellophane membranes (before). The cellophane with the fungal colony was removed and plates were incubated for an additional day to determine the presence of mycelial growth on the plate, indicative of cellophane penetration (after). (c) Apple slices were spot-inoculated with the indicated strains and imaged after 3 days of incubation at 28°C and 100% humidity. (d) Hyphal aggregates forming 48 h after inoculation of the indicated strains in liquid MM supplemented with $NaNO_3$, with or without 2 ng/ml rapamycin (R). Cultures were vortexed to dissociate weakly adhered hyphae and images were taken with a binocular microscope.

gtr1^{GTP}, and was fully rescued by rapamycin (Figure 5d). Collectively, these results demonstrate that TORC1 acts as a negative regulator of virulence-related functions such as invasive hyphal growth.

2.5 | Regulation of TORC1 is essential for virulence in *F. oxysporum*

We next asked whether correct modulation of TORC1 signalling is important for the ability of *F. oxysporum* to infect tomato plants. Tomato seedlings, whose roots were inoculated with microconidia of the wild type or the complemented *tsc2*Δ^{C} strain, developed progressive wilt symptoms and were mostly dead around 25 days postinoculation (dpi) (Figures 6a and S6). On the contrary, plants inoculated with *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ displayed much milder symptoms and a markedly reduced mortality rate. Furthermore, the fungal burden in roots and stems at 10 dpi was significantly lower in plants inoculated with *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ compared to plants inoculated with the wild type or the *tsc2*Δ^{C} strain. Interestingly, plants inoculated with *gtr1*^{GTP} also exhibited a reduction in mortality. However, in contrast to plants inoculated with *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ, those inoculated with *gtr1*^{GTP} contained more fungal biomass than those infected with the wild-type strain (Figure 6a,b).

Because *F. oxysporum* can cause disseminated infections in humans (Nucci & Anaissie, 2007), we tested the virulence of the different strains in larvae of the wax moth *Galleria mellonella*, an invertebrate model that is widely used to study microbial pathogens of humans, including *F. oxysporum* (Navarro-Velasco et al., 2011). Inoculation with microconidia of the wild type or the complemented *tsc2*Δ^{C} strain resulted in killing of all larvae at 3–5 dpi (Figure 6c). However, animals inoculated with *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ, and to a lesser extent those inoculated with *gtr1*^{GTP}, showed significantly lower mortality rates. Moreover, similar to the plant infection assays, the fungal burden at 2 dpi was lower in *G. mellonella* larvae infected with the *tsc2*Δ and *gtr1*^{GTP} *tsc2*Δ mutants and higher in those infected with *gtr1*^{GTP}, as compared to larvae infected with the wild type and the *tsc2*Δ^{C} strain (Figure 6d). Altogether, these results establish an important function of correct regulation of TORC1 during *F. oxysporum* infection on plant and animal hosts.

3 | DISCUSSION

During the infection process, fungal phytopathogens sense an array of environmental signals and must generate the appropriate developmental and morphogenetic responses to ensure successful colonization of the plant host (Perez-Martin et al., 2016). It has been proposed that nitrogen limitation acts as a key signal in plant pathogens to trigger the expression of virulence genes (López-Berges et al., 2010a; Snoeijers et al., 2000). Because TORC1 promotes cell growth and proliferation in response to nutrient sufficiency (Shertz et al., 2010), we hypothesized that uncontrolled activation of the TORC1 pathway should negatively impact virulence in *F. oxysporum*. Indeed, previous work in *M. oryzae* showed that mutations affecting carbon and nitrogen metabolism, which cause inappropriate activation of the TORC1 pathway, also impair appressorium formation (Marroquin-Guzman et al., 2017; Marroquin-Guzman & Wilson, 2015).

We attempted constitutive activation of the TORC1 signalling pathway in *F. oxysporum* by combining two different genetic approaches: (1) inactivation of the negative regulator Tsc2 resulting in overstimulation of the positive TORC1 regulator Rheb (Matsumoto et al., 2002; van Slegtenhorst et al., 2004) and (2) expression of a GTP-locked allele (*gtr1*^{GTP}) of the positive TORC1 regulator Gtr1 (Nicastro et al., 2017).

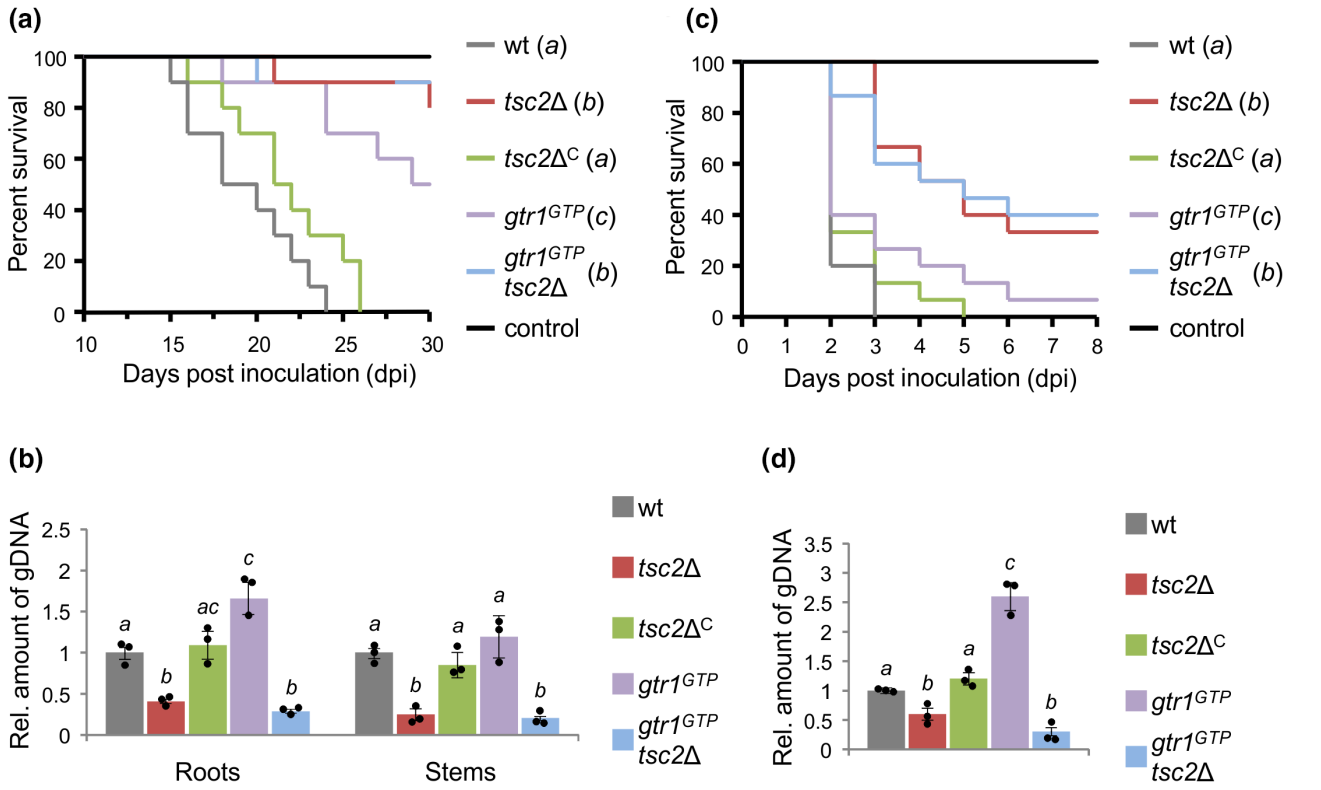

FIGURE 6 TORC1 regulation is important for virulence of *Fusarium oxysporum* on plant and animal hosts. (a, c) Kaplan–Meier plots showing the survival of tomato plants (a) or *Galleria mellonella* larvae (c) inoculated with the indicated fungal strains. Number of independent experiments = 3; 10 plants or 15 larvae per treatment. Data shown are from one representative experiment. Treatments with the same letter are not significantly different ($p < 0.05$) according to log-rank test. (b, d) Quantitative PCR was used to measure the relative amount of fungal DNA in total genomic DNA extracted from tomato roots and stems 10 days after inoculation (b) or from *G. mellonella* larvae 2 days after inoculation (d). Fungal burden is expressed relative to that measured in plants or larvae infected with the wild-type strain. Values with the same letter are not significantly different ($p < 0.05$) according to unpaired *t* test. Bars represent standard deviations calculated from three independent experiments with two replicates each.

We found that the first strategy indeed resulted in nutrient status-independent activation of TORC1 signalling, based on transcription of ribosomal genes and phosphorylation of Rps6, two readouts commonly used to measure TORC1 activation status (Gonzalez et al., 2015; Hannan et al., 2003; Martin et al., 2004; Sato et al., 2009). By contrast, expression of $gtr1^{GTP}$ in *F. oxysporum* had no detectable effect on TORC1 activation. A possible explanation is provided by the previous finding that expression of $gtr1^{GTP}$ in *S. cerevisiae* only leads to partial activation of TORC1 (Li & Guan, 2009). Moreover, recent work in *S. pombe* suggests that the role of Rag GTPases is more complex than previously recognized and also involves attenuation, rather than activation, of TORC1 (Chia et al., 2017; Fukuda et al., 2021; Fukuda & Shiozaki, 2018). Our data suggest that deletion of *tsc2* is the most effective approach for constitutive activation of TORC1 in *F. oxysporum*.

3.1 | Constitutive activation of TORC1 alters growth and stress response of *F. oxysporum*

To adapt growth to different environmental situations, fungi exquisitely regulate nutrient uptake and utilization. Under nutrient-limiting conditions, TORC1 is turned off and the production of transport proteins for nitrogenous compounds increases (Gonzalez & Hall, 2017; Segreto et al., 2021). On the contrary, under optimal growth conditions transporters and permeases in the plasma membrane are down-regulated in a TORC1-dependent manner via ubiquitination and endocytosis (Bowman 2nd et al., 2022). Here we propose that reduced growth of the *tsc2*Δ mutants on nonpreferred nitrogen sources is a consequence of inappropriate TORC1 activation. This is supported by the finding that the growth differences between the *tsc2*Δ strains and the wild type were levelled when TORC1 was inhibited by rapamycin.

In addition to affecting growth, inappropriate activation of TORC1 also increased sensitivity of *F. oxysporum* to cell wall and high temperature stresses, and this effect was reversed by rapamycin. The fungal cell wall provides a rigid cellular border and represents the first line of defence against adverse environmental conditions. In *F. oxysporum*, the cell wall is mainly composed of glucans, chitin, and glycoproteins (Schoffelmeer et al., 1999) and its integrity is essential for development and virulence (Fernandes et al., 2021; Martin-Udiroz et al., 2004; Nunez-Rodriguez et al., 2020). Interestingly, *S. cerevisiae* cells expressing a hyperactive *TOR1* allele were impaired

in β-1-3-glucan synthesis and exhibited increased sensitivity to cell wall-perturbing compounds (Ahmed et al., 2019). Furthermore, in *F. graminearum* loss of Sit4, a phosphatase that is inactivated by TORC1, caused increased sensitivity to cell wall-damaging agents and to high temperature stress (Yu et al., 2014). Interestingly, *F. oxysporum* mutants in the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) cascade also display increased sensitivity to both cell wall and high temperature stress (Segorbe et al., 2017). Although the functional link between CWI and TORC1 signalling pathways is not fully understood, a crosstalk between these two pathways has previously been suggested (Ahmed et al., 2019; Madrid et al., 2016; Sanchez-Adria et al., 2022; Yu et al., 2014).

Treatment of *F. oxysporum* germlings with the chitin-binding dye CFW produced intense staining of the cell wall and septae as previously reported (Martin-Udiroz et al., 2004), whereas in the *tsc2*Δ strains an abnormal punctate pattern of chitin deposition was observed. This was concomitant with a marked delay in germ tube growth. Abnormal chitin aggregates have also been observed in *S. cerevisiae* cells expressing a hyperactive *TOR1* allele (Ahmed et al., 2019). Because correct recycling of the chitin synthase Chs3 is required for polarized hyphal growth (Knafler et al., 2019), we speculate that constitutive activation of TORC1 could interfere with this process, as shown for other proteins localized in the plasma membrane (Bowman 2nd et al., 2022), thereby affecting cell wall biogenesis and germination. Importantly, chemical inhibition of TORC1 fully restored wild-type cell wall biogenesis and germination in the *tsc2*Δ mutants. We further found that microconidia production is increased in the *tsc2*Δ strains while biomass production is reduced. Previously, inactivation of the protein phosphatases Sit4 or Ppg1, both of which are inactive when TORC1 signalling is on, was also shown to increase conidiation and reduce mycelial growth in *F. graminearum* (Yu et al., 2014).

3.2 | TORC1 inhibits fungal virulence functions

Cellophane penetration is routinely used as a measure of invasive hyphal growth in *F. oxysporum* due to its similarities with the agar invasion assay in yeast (Gimeno et al., 1992) and because it highly correlates with the virulence phenotype on tomato plants (López-Berges et al., 2010a; Perez-Nadales & Di Pietro, 2011; Prados Rosales & Di Pietro, 2008). Cellophane penetration, as well as other virulence-related processes such as vegetative hyphal fusion or invasive growth on apple slices, were markedly inhibited in the *tsc2*Δ strains, while rapamycin-mediated TORC1 inactivation restored these functions. Together with previous evidence (Bastidas et al., 2009; López-Berges et al., 2010a, 2010b), our findings suggest a broadly conserved role of TORC1 as a negative regulator of infection-related processes in plant and human fungal pathogens. The finding that *F. oxysporum* mutants lacking *tsc2* are significantly reduced in their ability to invade and kill tomato plants or the non-vertebrate animal model *G. mellonella*, further highlights the relevance of the TORC1 pathway in fungal pathogenicity. The fact that inactivation of Tsc2 attenuates virulence of *F. oxysporum* on both plants and animals suggests that TORC1-regulated mechanisms are relevant for infection in both types of hosts. Previous studies established that both the invasive growth MAPK Fmk1 and the CWI MAPK Mpk1 control critical steps during the infection process of *F. oxysporum* (Di Pietro et al., 2001; Fernandes et al., 2021; Segorbe et al., 2017; Turra et al., 2014, 2015). Collectively, these findings suggest that the virulence phenotypes observed on TORC1 deregulation might be, at least in part, associated with defects in MAPK signalling. In line with this, inactivation of the protein phosphatase Sit4, which is negatively regulated by TORC1, increases cell wall stress and reduces virulence in *F. graminearum* (Yu et al., 2014). Both Sit4 and another phosphatase called Ppg1 interact with protein phosphatase Msg5, a negative regulator of the CWI MAPK pathway (Yu et al., 2014). Importantly, Msg5 was recently shown to regulate cell wall integrity and virulence in *F. oxysporum* (Fernandes et al., 2021).

Somewhat unexpectedly, expression of a $gtr1^{GTP}$ allele, which was previously shown to activate TORC1 in different organisms (Binda et al., 2009; Efeyan et al., 2013), was ineffective in *F. oxysporum* because no constitutive TORC1 activation was detected under the experimental conditions used in this study. Interestingly, however, the $gtr1^{GTP}$ mutant displayed some striking phenotypes such as increased proliferation as determined by higher fungal biomass in liquid cultures and under infection conditions, as well as attenuated virulence on plant and animal hosts. The discrepancy in phenotypes between the *tsc2*Δ and the $gtr1^{GTP}$ mutants in most assays carried out in this study (growth, stress, development, conidiation, invasive growth) suggest that the mechanisms underlying the virulence defects of these two mutants could be different. Importantly, inactivation of Tsc2 in a $gtr1^{GTP}$ background recapitulated all the phenotypes of the *tsc2*Δ single mutant and no intermediate phenotypes or additive effects were detected, suggesting that targeted deletion of *tsc2* is epistatic to $gtr1^{GTP}$ and that both components function in the same pathway.

In summary, our results support a conserved role of TORC1 as a negative regulator of fungal virulence. Moreover, the finding that fungal infection can be targeted by inappropriate TORC1 activation opens new avenues for antifungal discovery.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal isolates and culture conditions

F. oxysporum f. sp. *lycopersici* race 2 isolate Fol4287 (FGSC 9935) and the derived isogenic mutants were stored as microconidial suspensions at −80°C in 30% glycerol. For the extraction of genomic DNA and microconidia production, cultures were grown in PDB at 28°C (Di Pietro & Roncero, 1998). For the determination of colony growth, 2×10^4 microconidia were spotted onto PDA, minimal medium (Puhalla, 1985) with 25 mM of the indicated nitrogen source, or yeast extract peptone glucose (YPG) agar. When needed, media were supplemented with the indicated concentrations of rapamycin

(R) or calcofluor white (CFW) (both from Sigma-Aldrich). Cultures were incubated at the indicated temperatures for the specified time periods. Conidiation was quantified in YPG or in 25 mM sodium nitrate minimal medium ($NaNO_3$) static liquid cultures grown as described (Li et al., 2006; López-Berges et al., 2013). For fungal biomass quantification, 2.5×10^6 microconidia/ml were inoculated in YPG or $NaNO_3$ and cultures were maintained under standard conditions (28°C and 170 rpm). After 48 h the mycelium was harvested, dried, and weighed. Invasion assays on cellophane membranes (Colourless; Manipulados Margok) were performed as described (López-Berges et al., 2010a; Prados Rosales & Di Pietro, 2008) using solid minimal medium supplemented with 50 mM $NaNO_3$ with or without 2 ng/ml rapamycin. For macroscopic analysis of hyphal fusion and agglutination, fungal strains were grown for 48 h in minimal medium supplemented with 50 mM $NaNO_3$ with or without 2 ng/ml rapamycin. Cultures were vortexed to dissociate weakly adhered hyphae and aliquots were transferred to 12-well cell culture plates and imaged using a DFC 300 FX (Leica) digital camera coupled to a Leica binocular microscope driven by IM50 4.0 software (Leica). ImageJ software (National Institutes of Health) was used for contrast adjustment. For gene expression analysis, freshly obtained microconidia were germinated for 16 h in PDB. Germlings were harvested by filtration, washed three times in sterile water and transferred to (a) nitrogen-free minimal medium (−N) for 1 h before being shifted to −N or glutamine minimal medium (Gln) for an additional hour, (b) Gln for 1 h before being shifted to −N or −N + 100 ng/ml rapamycin (−N + R) for an additional hour, or (c) to Gln for 1 h before being shifted to Gln or Gln + 100 ng/ml rapamycin (Gln + R) for an additional hour. All experiments included three replicates and were performed at least twice with similar results.

4.2 | Generation of mutant strains

PCRs were routinely performed with VELOCITY DNA Polymerase (Bioline) using an MJ Mini personal thermal cycler (Bio-Rad). All fungal transformations and purification of the transformants by monoconidial isolation were performed as described previously (Di Pietro & Roncero, 1998). Targeted replacement of the entire coding region of the *F. oxysporum tsc2* gene was performed as depicted (Figure S2) using the split-marker method. Plasmid pAN7-1, containing the *hygromycin B resistance* gene (*hyg*) under the control of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator (Punt et al., 1987) was used. Transformants were genotyped by PCR (not shown) and Southern blot analysis, and the *tsc2*Δ#6 mutant was used for further experiments and complementation (Figure S2). Complementation of *tsc2*Δ with a PCR fragment encompassing the *tsc2* wild-type allele was done by cotransformation with the *phleomycin B resistance* gene under the control of the *A. nidulans gpdA* promoter and *trpC* terminator amplified from plasmid pAN8-1. Several phleomycin-resistant cotransformants were analysed for the presence of a functional *tsc2* gene and among them we specially selected those who had lost hygromycin resistance, therefore with *tsc2* integrated at its original locus. Cotransformants were genotyped by PCR (not shown) and Southern blot analysis, and $tsc2\Delta^{C}$#1 was selected for further experiments (Figure S2).

For the generation of a strain in which Gtr1 is expressed in a constitutively active (GTP-bound) form ($gtr1^{GTP}$), a $gtr1^{Q86L}$ allele was constructed by site-directed mutagenesis (Figure S4). Briefly, specific primers harbouring the $gtr1^{256CTA258}$ mutation (Figure S4) were used to amplify two fragments that were subsequently assembled by a fusion PCR method (Szewczyk et al., 2006). The obtained DNA fragment containing the $gtr1^{Q86L}$ allele was cloned into the pGEM-T vector (Promega), verified by Sanger sequencing, and used to cotransform protoplasts of the *F. oxysporum* wild-type strain together with the *phleomycin B resistance* gene. Several phleomycin-resistant transformants were analysed by amplification and sequencing of the relevant *gtr1* fragment. The fluorogram of $gtr1^{GTP}$#1, showing a discrete thymine (T) peak in position 257, indicates the sole presence of the $gtr1^{Q86L}$ allele in this strain (Figure S4c). The expression of exclusively the $gtr1^{Q86L}$ allele was further confirmed by reverse transcription (RT)-PCR and sequencing (not shown). Targeted replacement of *tsc2* in a $gtr1^{GTP}$ background was performed as described above using the split-marker method. Transformants were genotyped by PCR (not shown) and Southern blot analysis (Figure S5).

4.3 | Nucleic acid manipulations and RT-quantitative PCR

Total RNA and gDNA were extracted from *F. oxysporum* mycelia following previously reported protocols (Chomczynski & Sacchi, 1987; Raeder & Broda, 1985). Quality and quantity of the extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in a ND-1000 spectrophotometer (NanoDrop Technologies). Routine nucleic acid manipulations were performed according to standard protocols (Sambrook & Russell, 2001). RT-quantitative PCR (RT-qPCR) was performed as described previously (López-Berges et al., 2010a, 2012) using FastStart Essential DNA Green Master (Roche Diagnostics) in a CFX Connect real-time system (Bio-Rad). Transcript levels were calculated by comparative ΔC_t and normalized to *act1*.

4.4 | Western blot

Proteins were extracted using a reported procedure (Hervas-Aguilar & Penalva, 2010; López-Berges et al., 2016) involving solubilization from lyophilized mycelial biomass with NaOH, followed by precipitation with trichloroacetic acid (TCA). Aliquots were resolved in 10%–12% SDS-polycrylamide gels and transferred to nitrocellulose membranes with a Trans-Blot Turbo transfer system (Bio-Rad) for blotting. Western blots were reacted with Phospho-(Ser/Thr) Akt substrate antibody (1:10,000; #9611 Cell Signalling Technology) as primary and with anti-rabbit IgG-peroxidase antibody (1:10,000; A1949; Sigma-Aldrich) as secondary, or with

anti-α-tubulin antibody (1:10,000; T6119; Sigma-Aldrich) as primary and anti-mouse IgG-peroxidase antibody (1:10,000; A4416; Sigma-Aldrich) as secondary. Proteins were detected with ECL (Amersham Biosciences).

4.5 | Fluorescence microscopy

F. oxysporum microconidia were germinated in PDB with or without 2 ng/ml rapamycin for 2 h or 6–8 h and stained with 6 mg/ml CFW. Images were acquired at 100× magnification using a DFC 300 FX digital camera coupled to a DMR microscope driven by IM50 4.0 software (Leica). ImageJ software was used for contrast adjustment.

4.6 | Infection assays

Tomato root inoculation assays were performed as described (Di Pietro & Roncero, 1998) using 2-week-old tomato seedlings, cultivar Monika (Syngenta). Severity of disease symptoms and plant survival were recorded daily for 30 dpi. Ten plants were used for each treatment. Data were analysed with GraphPad Prism 5 software. Quantification of fungal biomass in planta was performed as described in Pareja-Jaime et al. (2010) using total gDNA extracted from tomato roots or stems infected with *F. oxysporum* strains at 10 dpi. Relative amounts of fungal gDNA were calculated by comparative ΔC_t of the *F. oxysporum act1* gene normalized to the tomato *EF1α* gene. Infection assays on apple slices, cultivar Golden Delicious, were performed as described (López-Berges et al., 2009). Colonization and maceration of the fruit tissue were monitored daily for 3–5 dpi. *G. mellonella* infection assays were performed as described (Navarro-Velasco et al., 2011). *G. mellonella* larvae (Nutri-reptil) were maintained in plastic boxes for 2–3 days before the infection. Fifteen larvae were used for each treatment. An automicroapplicator (0.1–10 μl; Burkard Manufacturing Co. Ltd) with a 1 ml syringe (Terumo Medical Corporation) was used to inject 8 μl of a 1.6×10^5 microconidial suspension into the haemocoel of each larva. After injection, larvae were incubated in glass containers at 30°C. Survival was recorded daily for 10 dpi. Data were analysed with GraphPad Prism 5 software. Quantification of fungal biomass in *G. mellonella* larvae was performed as described using total gDNA extracted from animals infected with *F. oxysporum* strains at 2 dpi. Relative amounts of fungal gDNA were calculated by comparative ΔC_t of the *F. oxysporum act1* gene normalized to the *G. mellonella gallerimycin* gene. Virulence experiments were performed at least three times with comparable results.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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