

Host-induced gene silencing of *BcTOR* in *Botrytis cinerea* enhances plant resistance to grey mould

FANGJIE XIONG^{1,2,#}, MEI LIU^{1,2,#}, FENGPING ZHUO^{1,2,3,#}, HUAN YIN^{1,2}, KEXUAN DENG^{1,2}, SHUN FENG^{1,2}, YUDONG LIU^{1,2}, XIUMEI LUO^{1,2}, LI FENG^{1,2}, SHUMIN ZHANG^{1,2}, ZHENG GUO LI^{1,2} AND MAO ZHI REN^{1,2,4,*}

¹School of Life Sciences, Chongqing University, Chongqing 401331, China

²Key Laboratory of Plant Hormone and Developmental Regulation of Chongqing, Chongqing 401331, China

³School of Chemistry and Chemical Engineering, Chongqing University of Science and Technology, Chongqing 401331, China

⁴Institute of Urban Agriculture, Chinese Academy of Agricultural Sciences/National Chengdu Agricultural Science and Technology Center, Chengdu 610000, China

SUMMARY

Botrytis cinerea is the causal agent of grey mould for more than 200 plant species, including economically important vegetables, fruits and crops, which leads to economic losses worldwide. Target of rapamycin (TOR) acts a master regulator to control cell growth and proliferation by integrating nutrient, energy and growth factors in eukaryotic species, but little is known about whether TOR can function as a practicable target in the control of plant fungal pathogens. Here, we characterize TOR signalling of *B. cinerea* in the regulation of growth and pathogenicity as well as its potential value in genetic engineering for crop protection by bioinformatics analysis, pharmacological assays, biochemistry and genetics approaches. The results show that conserved TOR signalling occurs, and a functional FK506-binding protein 12 kD (FKBP12) mediates the interaction between rapamycin and *B. cinerea* TOR (BcTOR). RNA sequencing (RNA-Seq) analysis revealed that BcTOR displayed conserved functions, particularly in controlling growth and metabolism. Furthermore, pathogenicity assay showed that BcTOR inhibition efficiently reduces the infection of *B. cinerea* in plant leaves of *Arabidopsis* and potato or tomato fruits. Additionally, transgenic plants expressing double-stranded RNA of *BcTOR* through the host-induced gene silencing method could produce abundant small RNAs targeting *BcTOR*, and significantly block the occurrence of grey mould in potato and tomato. Taken together, our results suggest that BcTOR is an efficient target for genetic engineering in control of grey mould, and also a potential and promising target applied in the biocontrol of plant fungal pathogens.

Keywords: *Botrytis cinerea*, host-induced gene silencing, mycelial growth, pathogenicity, target of rapamycin.

*Correspondence: Email: renmaozhi@cqu.edu.cn

#These authors contributed equally to this work.

INTRODUCTION

Botrytis cinerea, a widespread necrotrophic fungal plant pathogen that can infect more than 200 plant species, including economically important vegetables, fruits and crops, and lead to grey mould rot or botrytis blight, causes widespread losses every year worldwide (Choquer *et al.*, 2007; Dean *et al.*, 2012; Williamson *et al.*, 2007). More than 40% of these losses occur in greenhouse-grown and field-grown crops if chemical control is not used (Pedras *et al.*, 2011; Villa-Rojas *et al.*, 2012). Thus, chemical pesticides are widely required for the control of *B. cinerea*. However, the widespread use of chemical pesticides results in serious water, soil, food and environmental pollution (Malhat *et al.*, 2015; Oliveira *et al.*, 2015; Tomenson and Matthews, 2009). To overcome this issue, biofungicides with low toxicity and high efficiency against key targets of pathogens have been developed and applied to control plant fungal disease. In addition, genetic engineering methods have also been extensively employed to improve resistance to plant pathogens in crop protection. For example, host-induced gene silencing (HIGS), an RNA interference (RNAi)-based approach in which small RNAs (sRNAs) are produced by the host plant to target invader transcripts, has emerged as an effective strategy for improving plant resistance against pathogens (Cai *et al.*, 2018; Wang, Thomas *et al.*, 2017).

HIGS has been widely used for crop protection and a wide range of transgenic crops acquire durable resistance against diseases by expressing double-stranded RNA (dsRNA) that is subsequently processed into sRNAs targeting essential genes regulating growth or pathogenicity (Cheng *et al.*, 2015; Ghag *et al.*, 2014; Govindarajulu *et al.*, 2015; Koch *et al.*, 2013; Nowara *et al.*, 2010; Nunes and Dean, 2012; Xu *et al.*, 2018; Zhang *et al.*, 2016; Zhu *et al.*, 2017). For instance, engineered corn plants expressing a vacuolar ATPase (V-ATPase) dsRNA can be protected from western corn rootworm feeding damage; durable resistance to Fusarium head blight and seedling blight can be acquired by

wheat through HIGS targeting to an essential chitin synthase gene *Chs3b*; and the virulence genes of *Verticillium dahliae* can be silenced by plant-mediated RNAi, compromising verticillium wilt in tomato and *Arabidopsis* (Baum *et al.*, 2007; Cheng *et al.*, 2015; Song and Thomma, 2018). In a study to control *B. cinerea*, *Arabidopsis* plants with hairpin RNAs simultaneously targeting dicer-like genes of *B. cinerea* and *V. dahliae* exhibited enhanced resistance to both pathogens (Wang *et al.*, 2016).

Target of rapamycin (TOR), a Ser/Thr protein kinase of large molecular weight with multiple functional domains, is a well-known essential gene in eukaryotic species, but little is known about whether or not TOR can be developed and established as a key target for biological control of plant fungal pathogens. TOR was first discovered in yeast (*Saccharomyces cerevisiae*) by a genetic screen of mutants insensitive to rapamycin (Heitman *et al.*, 1991). Rapamycin, a well-known medicine produced by *Streptomyces hygroscopicus*, is able to efficiently repress TOR kinase activity. FK506 binding protein 12 kD (FKBP12), as a receptor of rapamycin, mediates inhibition of TOR by rapamycin and its defects confer resistance to rapamycin (Heitman *et al.*, 1991). In the presence of rapamycin, FKBP12 forms a binary complex with rapamycin and further interacts with the FKBP12-rapamycin binding domain (FRB) of TOR to form a ternary complex, resulting in abolishment of TOR activity (Chiu *et al.*, 1994; Heitman *et al.*, 1991; Koltin *et al.*, 1991; Sabatini *et al.*, 1994; Vezina *et al.*, 1975). Based on the rapamycin-FKBP12-TOR system, the TOR signalling pathway has been extensively studied and gradually elucidated in yeast and mammals (Benjamin *et al.*, 2011). The TOR protein consists of five highly conserved domains: HEAT repeats (Huntingtin, elongation factor 3 (EF3), a subunit of protein phosphatase 2A (PP2A) and TOR1), a FAT (FRAP, ATM and TRRAP) domain, a FRB (FKBP12-rapamycin binding) domain, a kinase and a FATC (carboxy-terminal FAT) domain, which reside in the TOR protein between the N-terminal and the C-terminal (Baretic and Williams., 2014; Sauer *et al.*, 2013; Yang *et al.*, 2013). Most eukaryotic organisms have one copy of the *TOR* gene, but two and three *TOR* genes have also been identified in yeast and *Leishmania major*, respectively (Heitman *et al.*, 1991; Madeira da Silva and Beverley, 2010). In some eukaryotes, based on diverse components recruited by TOR, the TOR protein can form two different types of multiprotein complex: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). The TOR protein recruits a regulatory-associated protein of TOR (RAPTOR) and a lethal with SEC13 protein8 (LST8) to form TORC1, but combines LST8, stress-activated map kinase-interacting protein 1 (SIN1) and rapamycin-insensitive companion of TOR (RICTOR) to form TORC2 in yeast and animals (Loewith *et al.*, 2002; Wullschleger *et al.*, 2006). Due to this difference, TORC1 is sensitive to rapamycin, but TORC2 shows insensitivity to rapamycin (Loewith *et al.*, 2002). In addition, rapamycin-sensitive TORC1 plays a

major role in cell growth, development and proliferation in a temporal manner, while rapamycin-resistant TORC2 seems to particularly regulate the development of the cell cytoskeleton (Feldman *et al.*, 2009; Loewith *et al.*, 2002; Takahara and Maeda, 2013; Wang and Proud, 2009; Wullschleger *et al.*, 2006).

Various studies have shown that TOR is a central coordinator of energy, nutrient and stress signalling networks from yeast to mammals and plants (Dobrenel *et al.*, 2016; Henriques *et al.*, 2014; Loewith and Hall, 2011; Rexin *et al.*, 2015; Schmelzle and Hall, 2000; Xiong and Sheen, 2015). However, little attention has been paid to TOR signaling study in phytopathogenic fungi are relatively less studied. Rapamycin was discovered in the early 1970s as an antifungal agent against the pathogenic yeast *Candida albicans* (Sehgal *et al.*, 1975). Subsequent studies also found that rapamycin is effective against many phytopathogenic fungi, such as *Botrytis cinerea*, *Mucor circinelloides*, *Fusarium fujikuroi*, *Fusarium oxysporum* and *Fusarium graminearum* (Bastidas *et al.*, 2012; Lopez-Berges *et al.*, 2010; Melendez *et al.*, 2009; Teichert *et al.*, 2006; Yu *et al.*, 2014), indicating that a conserved TOR pathway also exists in plant pathogenic fungi. In addition, the research on TOR signalling in *F. graminearum* elucidated the TOR components and conserved functions in growth and development as well as its role in virulence (Yu *et al.*, 2014). In *B. cinerea*, it was reported that a functional FKBP12 homologous protein existed with conserved function in bridging rapamycin, and the BcFKBP12 deletion relieved growth inhibition of rapamycin and reduced virulence of the strain T4 while not affecting the pathogenic development of the strain B05.10 (Gioti *et al.*, 2006). Additionally, BcFKBP12 was reported to likely be involved in sulphur regulation and this regulation appears to be unrelated to TOR signalling (Melendez *et al.*, 2009). To date, information on the TOR signalling pathway in *B. cinerea* is very limited, and TOR function remains to be determined.

In the present study, we functionally characterized *B. cinerea* TOR (BcTOR) in the regulation of vegetative growth and development as well as virulence of the *B. cinerea* strain B05.10 by bioinformatics analysis, pharmacological assays and chemical genetics approaches. We created transgenic potato and tomato plants expressing dsRNA specific to *BcTOR* based on the HIGS method, and found that transgenic plants could produce abundant sRNA molecules targeting *BcTOR*, significantly blocking the occurrence of grey mould caused by *B. cinerea*. Our results suggest that BcTOR can function as a potential and promising target in the control of grey mould disease.

RESULTS

Molecular components of the TOR complex in *B. cinerea*

To investigate TOR signalling in *B. cinerea*, a yeast TOR protein sequence was used to search for the homologous sequences in the *B. cinerea* genome (http://fungi.ensembl.org/Botrytis_cinerea/

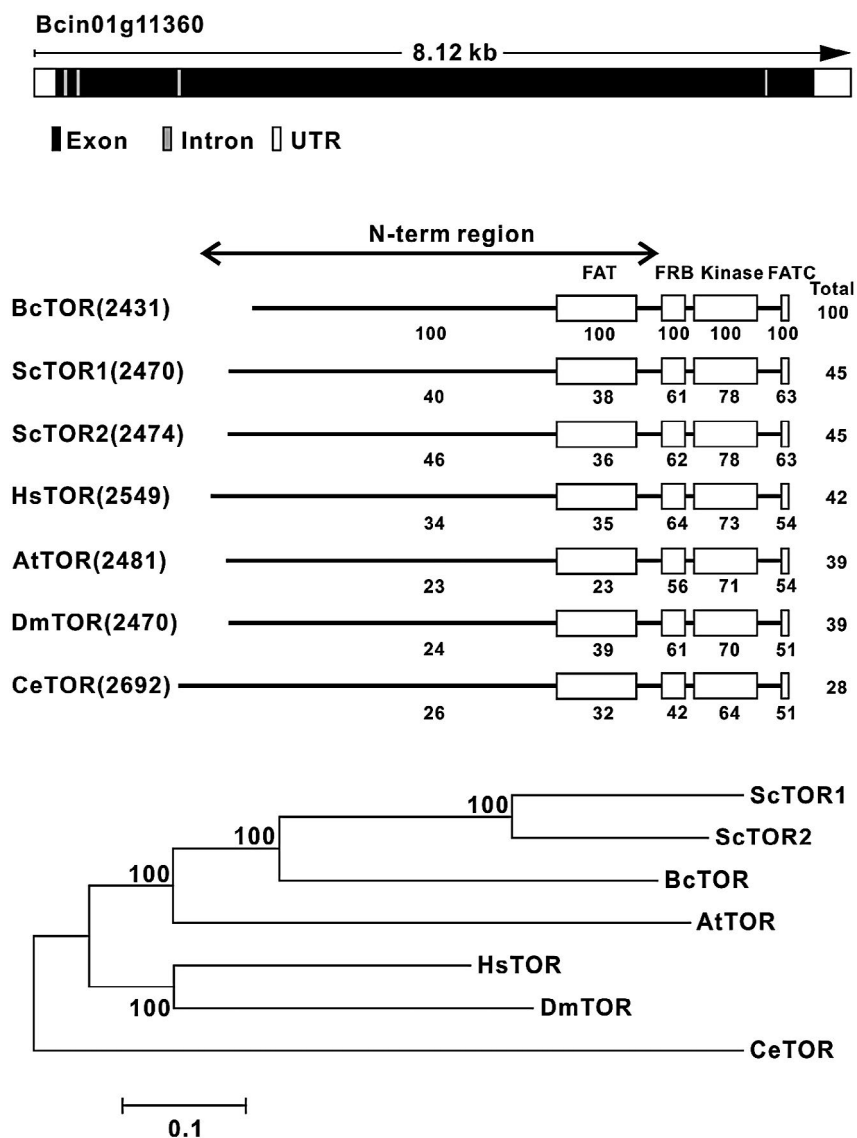


Fig. 1 The information for the TOR homologue in *Botrytis cinerea*. (A) The gene locus and structure of the TOR homologue in *B. cinerea*. (B) Domain organization of BcTOR protein and comparison of the BcTOR amino acid sequence with those of TOR proteins from other organisms. (C) Phylogenetic analysis of BcTOR with that from other species. Bc, *Botrytis cinerea*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*; At, *Arabidopsis thaliana*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*. Protein domain diagram shows number of HEAT (Huntingtin, elongation factor 3 (EF3), a subunit of protein phosphatase 2A (PP2A) and TOR1) repeats; FAT (FRAP, ATM and TRRAP) domain; FRB (FKP12-rapamycin binding (FRB) domain); and carboxy-terminal FAT (FATC) domain.

Info/Index) and NCBI (<http://www.ncbi.nlm.nih.gov/>), respectively. The search results revealed a single TOR homologue gene (named *BcTOR*), located on the first chromosome of the *B. cinerea* genome (Fig. 1A). Further analysis showed that the full-length genomic DNA of *BcTOR* spans about 8.12 kb and contains five exons and four introns in the *B. cinerea* genome (Fig. 1A), including a 7296 bp full-length coding sequence encoding a protein of 2431 amino acid residues with a predicted molecular mass of 275 kDa. Alignment of BcTOR with TOR protein sequences from other species showed similar domain organization with significant identification and conservation of the FRB and kinase domains as

well as the FATC domain at the C-terminus (Fig. 1B), which are conserved in the phosphatidylinositol 3-kinase related protein kinase family (Fruman and Rommel, 2014; Sauer *et al.*, 2013; Schmelzle and Hall, 2000; Yang *et al.*, 2013). Additionally, a conserved motif sequence named HEAT, reported to be involved in protein–protein interactions (Hara *et al.*, 2002; Schmelzle and Hall, 2000), was found to be distributed throughout the N-terminal region of BcTOR (Fig. 1B).

Phylogenetic analysis revealed that BcTOR is evolutionarily conserved and depicts a closer evolutionary relationship with ScTOR1/2 in comparison with other TORs (Fig. 1C). In yeast or

animals, TOR protein was reported to form two structurally and functionally distinct multiprotein complexes through recruiting shared and distinct TOR-interacting components (Helliwell *et al.*, 1994; Wullschleger *et al.*, 2006). RAPTOR and LST8, as the core components of TORC1, each depicted one homologous gene through homologous comparison in the *B. cinerea* genome (Table 1). Additionally, RICTOR, a specific component of TORC2, also was found in the *B. cinerea* genome (Table 1), illustrating the existence of TORC2. These results indicate the existence of conserved TOR signalling in *B. cinerea*.

BcFKBP12 rescues *Arabidopsis* sensitivity to rapamycin

Early studies demonstrated that the FKBP12 protein from yeast or humans could restore rapamycin sensitivity in *Arabidopsis* (Mahfouz *et al.*, 2006; Ren *et al.*, 2012; Sormani *et al.*, 2007; Xiong *et al.*, 2017). Our previous study also indicated that tomato FKBP12 could restore the inhibitory effect of rapamycin to TOR (Xiong *et al.*, 2016). Likewise, in *B. cinerea*, FKBP12 homologous protein was found with conserved function in bridging rapamycin and FK506, and it was also reported that *BcFKBP12* is involved in T4 strain virulence and sulphur regulation (Gioti *et al.*, 2006; Melendez *et al.*, 2009). Here, to further confirm BcFKBP12 function in TOR signalling, we cloned and expressed *BcFKBP12* in *Arabidopsis*. As shown in Fig. 2A, there was no change in morphological phenotypes observed in these transgenic lines as compared with wild-type (WT) plants. However, as expected, the transgenic lines expressing *BcFKBP12* showed sensitivity to rapamycin and displayed a retarded growth phenotype with small cotyledons, reduced biomass (fresh weight) and shorter primary roots than

that of WT under rapamycin treatment (Fig. 2A–E). In addition, this acquired response was dose-independent (Fig. 2D,2). Similar results were observed in *Arabidopsis* expressing *ScFKBP12*, while no difference was found in WT under rapamycin treatment in comparison to DMSO treatment (Fig. 2D,E). These observations further confirm the conserved function of BcFKBP12 in mediating the inhibitory effect of rapamycin on TOR activity.

BcTOR regulates mycelial growth and conidiation, but not conidial germination of *B. cinerea*

Rapamycin is used to study the TOR signalling pathway in eukaryotic species. Previous reports indicated that rapamycin is able to suppress growth mediated by the FKBP12 orthologue in *B. cinerea*, but there is a lack of detailed description about TOR function in these studies. Here we set up a series of concentrations of rapamycin from 1 nM to 1 µM to observe the inhibition morphology of *B. cinerea* caused by rapamycin. The results show that radial growth of *B. cinerea* is severely inhibited on potato dextrose agar (PDA) amended with rapamycin even at a very low dose (1 nM) and nearly ceases growth with about 98% inhibitory rate at 100 nM (Fig. 3A,B,D). Microscopic observation of hyphal morphology showed that hyphae treated with rapamycin had more branches with increasing dose of rapamycin compared with the solvent control (Fig. 3C). Furthermore, twisted hyphal morphology was observed (Fig. 3C), similar to a previous study in *F. graminearum* (Yu *et al.*, 2014). In accordance with retarded mycelial growth, there was a significant decline in biomass of *B. cinerea* when treated with rapamycin under liquid culture conditions (Fig. S1). These results suggest

Table 1 TORC1 and TORC2 homologues in *Botrytis cinerea* for various species

	Hs	Sc	Bc
TORC1	mTOR	TOR1/2	TOR (Bcin01g11360)
	RAPTOR	Kog1	RAPTOR (Bcin06g02850)
	LST8	Lst8	LST8 (Bcin04g05980)
	-	Toc89	-
	PRAS40	-	-
	DEPTOR	-	-
TORC2	mTOR	TOR2	TOR
	SIN1	Avo1	SIN1 (Bcin16g01100)
	-	Avo2	-
	RICTOR	Avo3	RICTOR (Bcin03g06820)
	LST8	Lst8	LST8
	PRR5	Bit61	-
	DEPTOR	-	-

Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Bc, *Botrytis cinerea*. TORC1, target of rapamycin complex 1; TORC2, target of rapamycin complex 2; mTOR, mammalian target of rapamycin; RAPTOR, regulatory-associated protein of mTOR; LST8, lethal with SEC13 protein8; SIN, stress-activated map kinase-interacting protein 1; RICTOR, rapamycin-insensitive companion of mTOR. (–) indicates that there are no shown/obvious homologues of the indicated proteins in the corresponding organisms.

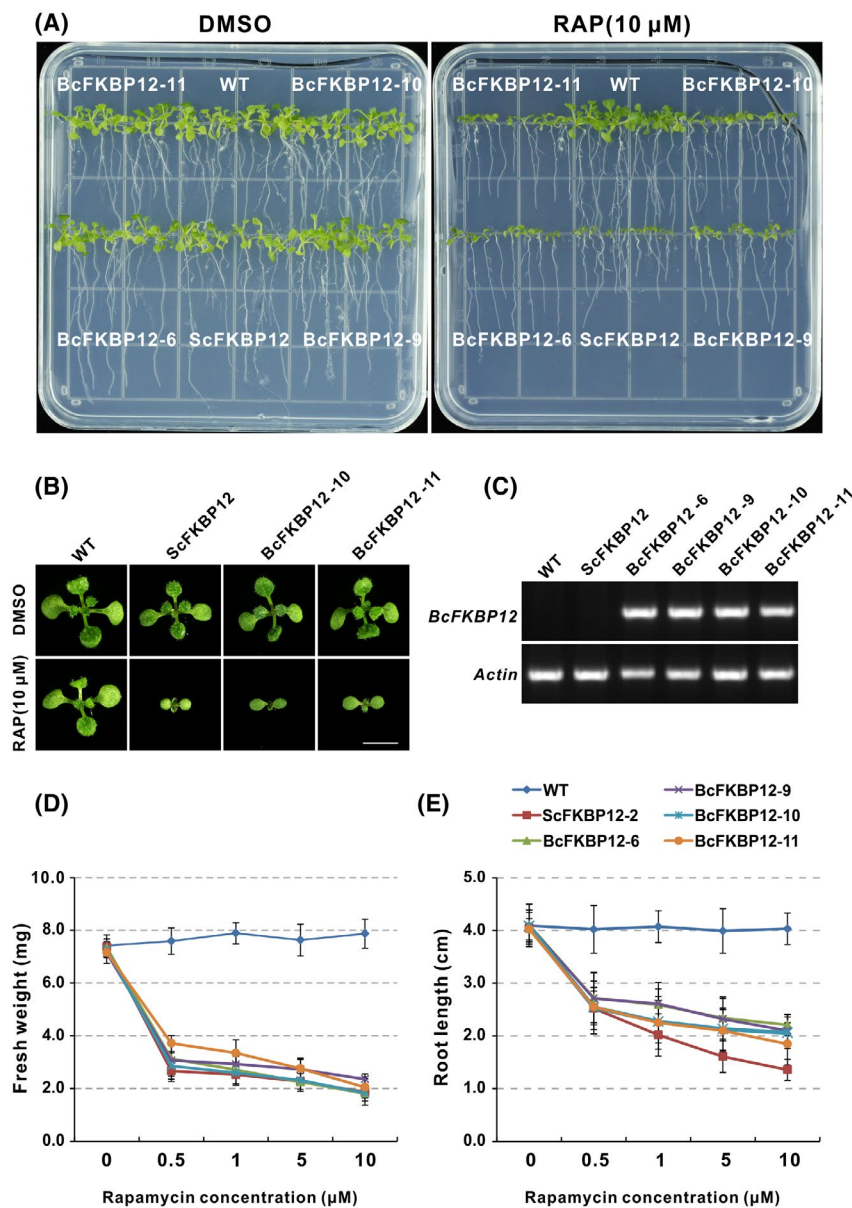


Fig. 2 *BcFKBP12* restores sensitivity of *Arabidopsis* to rapamycin. (A) Seedlings of *BcFKBP12*-expressing lines showed growth retardation like *ScFKBP12*-expressing plants under rapamycin treatment. (B) Cotyledon phenotype of wild-type (WT), *ScFKBP12*- and *BcFKBP12*-expressing plants under rapamycin treatment. Bar = 0.5 cm. (C) The detection of *BcFKBP12* in transcript levels. (D, E) Dosage-dependent curves of rapamycin for plant fresh weight and primary root length for different *Arabidopsis* plants measured at 10 days after germination. Error bars indicate SEM ($n \geq 40$). The experiment was repeated three times for each treatment. SEM, standard error of the mean.

that TOR plays an important role in regulating the vegetative growth of *B. cinerea*.

In addition, rapamycin also exhibited a strong inhibitory effect on sporulation. Microscopic observation showed twisted hyphae with few conidiophores on 10 nM rapamycin plates compared to the control plate (Fig. 4A). Furthermore, spore number statistics showed that there is no difference in spore production between treatment of 1 nM rapamycin and DMSO solvent, while conidium production

was dramatically decreased under 10 nM rapamycin treatment (Fig. 4B). The spores were not observed on PDA plates with 100 nM rapamycin (Fig. 4B). These results suggest that TOR activity is required for sporulation of *B. cinerea*. Subsequently, we investigated the effects of rapamycin on conidial germination through dropping spore suspensions on PDA plates supplemented with rapamycin or DMSO. As shown in Fig. 4C, after 3 days of incubation we observed smaller colonies on the rapamycin plates than on the DMSO plates.

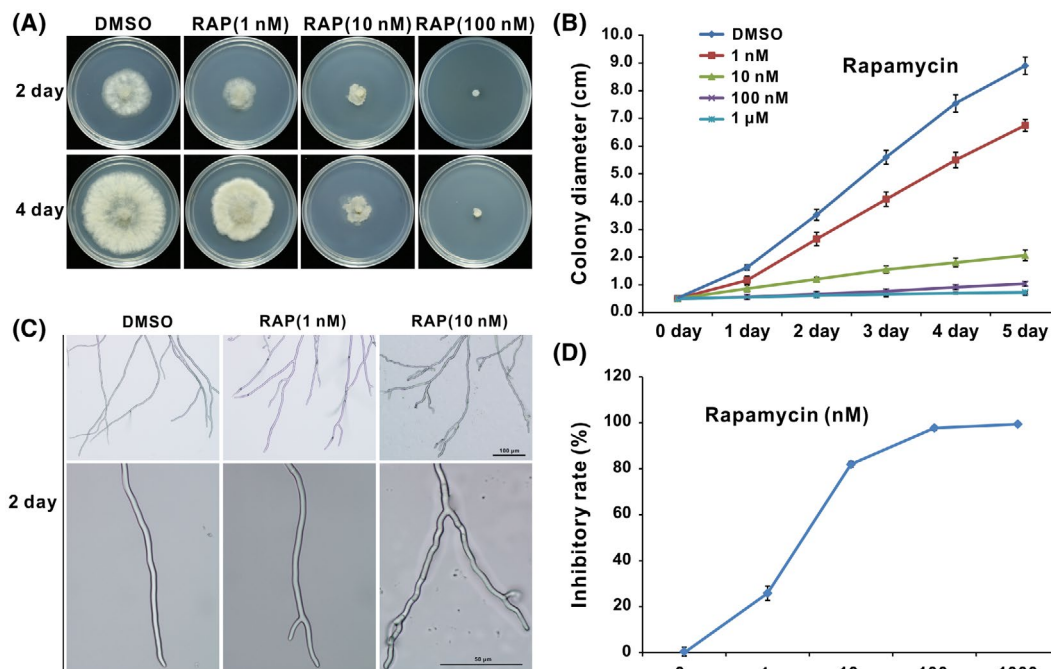


Fig. 3 TOR inhibition under rapamycin retards mycelial growth of *Botrytis cinerea*. (A) Inhibitory effect of rapamycin against *B. cinerea*. (B) Dosage-dependent curves of rapamycin for colony diameter of *B. cinerea* on potato dextrose agar plates. Error bars indicate the SEM ($n \geq 10$). (C) Hyphal morphology of *B. cinerea* under rapamycin treatment. (D) Inhibitory rate of rapamycin on mycelial growth of *B. cinerea*. Error bars indicate the SEM ($n \geq 10$). These experiments were repeated more than three times for each treatment.

However, there was almost no difference in conidial germination at different doses of rapamycin compared with DMSO when observed at indicated time points (Fig. 4D), suggesting BcTOR is not involved in regulating conidial germination of *B. cinerea*.

BcTOR inhibition reprograms gene expression to modulate growth and metabolism

To further investigate the role of BcTOR in the growth of *B. cinerea*, we performed whole genome expression profiling analysis on rapamycin and DMSO treatments through RNA sequencing (RNA-Seq). RNA-Seq data indicated that there were 3257 differentially expressed genes (DEGs), consisting of 1448 up-regulated DEGs and 1809 down-regulated DEGs (Fig. 5A and Table S1). Gene ontology (GO) enrichment analysis showed that these DEGs were categorized into 238 significantly enriched GO terms (q -value < 0.01) (Table S2). In the top 30 of the most significantly enriched GO terms, most were associated with transcription, translation and ribosome biogenesis (Fig. 5B), which all belong to the conserved function of TOR signalling in regulation of growth (Loewith and Hall, 2011; Mahoney *et al.*, 2009; Thoreen *et al.*, 2012; Wullschlegler *et al.*, 2006). Furthermore, the DEGs classified in these top 30 terms displayed an almost unanimous down-regulated trend (Fig. 5B and Table S2), suggesting that rapamycin represses BcTOR function in regulation of growth of *B. cinerea*. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway analysis showed that many DEGs were mainly enriched on 169 KEGG pathways, including 91 down-regulated pathways and 78 up-regulated pathways (Tables S3 and S4). In the top 20 most enriched pathways, the majority were involved in anabolism in down-regulated groups, such as 'ribosome' and 'ribosome biogenesis', which were the two most significantly enriched pathways (Fig. 5C and Table S3). Conversely, in the up-regulated top 20 enriched pathways, most participated in catabolism, such as 'valine, leucine and isoleucine degradation' and 'fatty acid degradation' (Fig. 5D and Table S4). These results show consistency with the conserved function of TOR in modulating metabolism (Robaglia *et al.*, 2012; Wullschlegler *et al.*, 2006). Taken together, the above data indicate there is a conserved TOR function in the regulation of growth and metabolism in *B. cinerea*.

BcTOR inhibition reduces infection by *B. cinerea*

To investigate whether TOR affects infection by *B. cinerea*, detached leaves of *Arabidopsis* and potato plants, as well as mature red tomato fruits from the supermarket, were drop-inoculated with *B. cinerea* spore suspension mixed with indicated doses of rapamycin. After 3 days of incubation, larger water-soaked infection lesions and more pathogen colonization were observed on leaves of both *Arabidopsis* and potato when infected with *B. cinerea* under DMSO treatment compared with that of rapamycin treatment (Fig. 6A–C). In particular, infected spots were

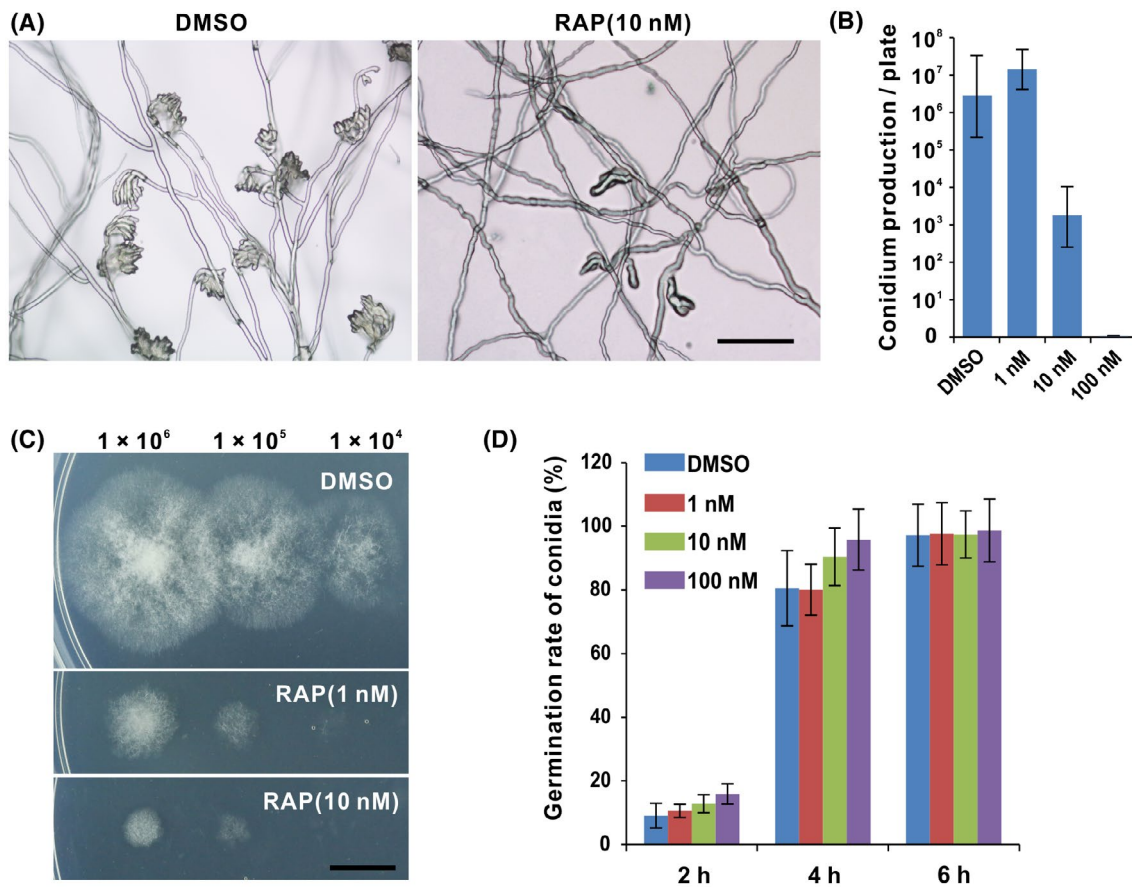


Fig. 4 TOR inhibition represses conidiation but not conidial germination of *Botrytis cinerea*. (A) TOR affects sporulation of *B. cinerea*. (B) Conidium production under TOR inhibition. Error bars indicate the SEM ($n = 10$). The experiments were repeated three times. (C) Mycelial growth after germination under TOR inhibition. Scale bar represents 1 cm. (D) Conidial germination rate was calculated at the indicated time points. Data are represented as means \pm SD ($n = 3$).

hardly observed in the case of treatment with 1 μ M of rapamycin (Fig. 6A). Similarly, tomato fruits showed smaller lesions and less pathogen biomass when infected with *B. cinerea* under rapamycin conditions at 7 days after incubation (Fig. 6A-C). Furthermore, after 15 days of incubation, tomato fruits exhibited severe decay covered by mycelium under the solvent conditions, while rapamycin efficiently reduced infection by *B. cinerea* and even blocked the infection at high concentrations (Fig. S2). These findings suggest that BcTOR is required for pathogenicity of *B. cinerea*.

BcTOR silencing by plant-mediated RNAi enhances resistance to *B. cinerea* in plants

HIGS has become a promising strategy in genetic engineering methods for crop protection. Transgenic plants and crops expressing dsRNAs that target essential growth and virulence genes of eukaryotic pathogens and pests are less prone to disease (Cai *et al.*, 2018; Nunes and Dean, 2012; Wang, Thomas *et al.*, 2017). Our previous results on BcTOR function showed that BcTOR could be a potential target in HIGS for crop protection. In order

to test its potential, three fragments of *BcTOR* (BcRI1, BcRI2 and BcRI3) were selected to construct the RNA interference vector, and transgenic potato was first generated due to the ease of genetic transformation (Fig. 7A). A total of 25 independent transgenic potato lines were generated, including six BcRI1 lines (BcRI1-1 to BcRI1-9), nine BcRI2 lines (BcRI2-1 to BcRI2-6) and ten BcRI3 lines (BcRI3-1 to BcRI3-10), in which several lines were selected for pathogen infection experiment (Fig. S3). As shown in Fig. 7B, the selected transgenic potato lines all showed enhanced resistance to *B. cinerea* to different extents, manifested in smaller lesions and less pathogen biomass compared with WT or green fluorescent protein (GFP) dsRNA-expressing plants (Fig. 7B-D). In particular, BcRI3-3 and BcRI3-6 lines displayed near-complete resistance to *B. cinerea* so water-soaked lesions were hardly observed (Fig. 7A). In order to confirm that the enhanced resistance of transgenic potato is related to *BcTOR* transcript level, we examined the silencing efficiency of *BcTOR* by qRT-PCR in these infected leaves. In accordance with the above observation, the transcript level of *BcTOR* was significantly decreased in the infected *BcTOR*-silenced transgenic lines in comparison with WT or GFP dsRNA-expressing

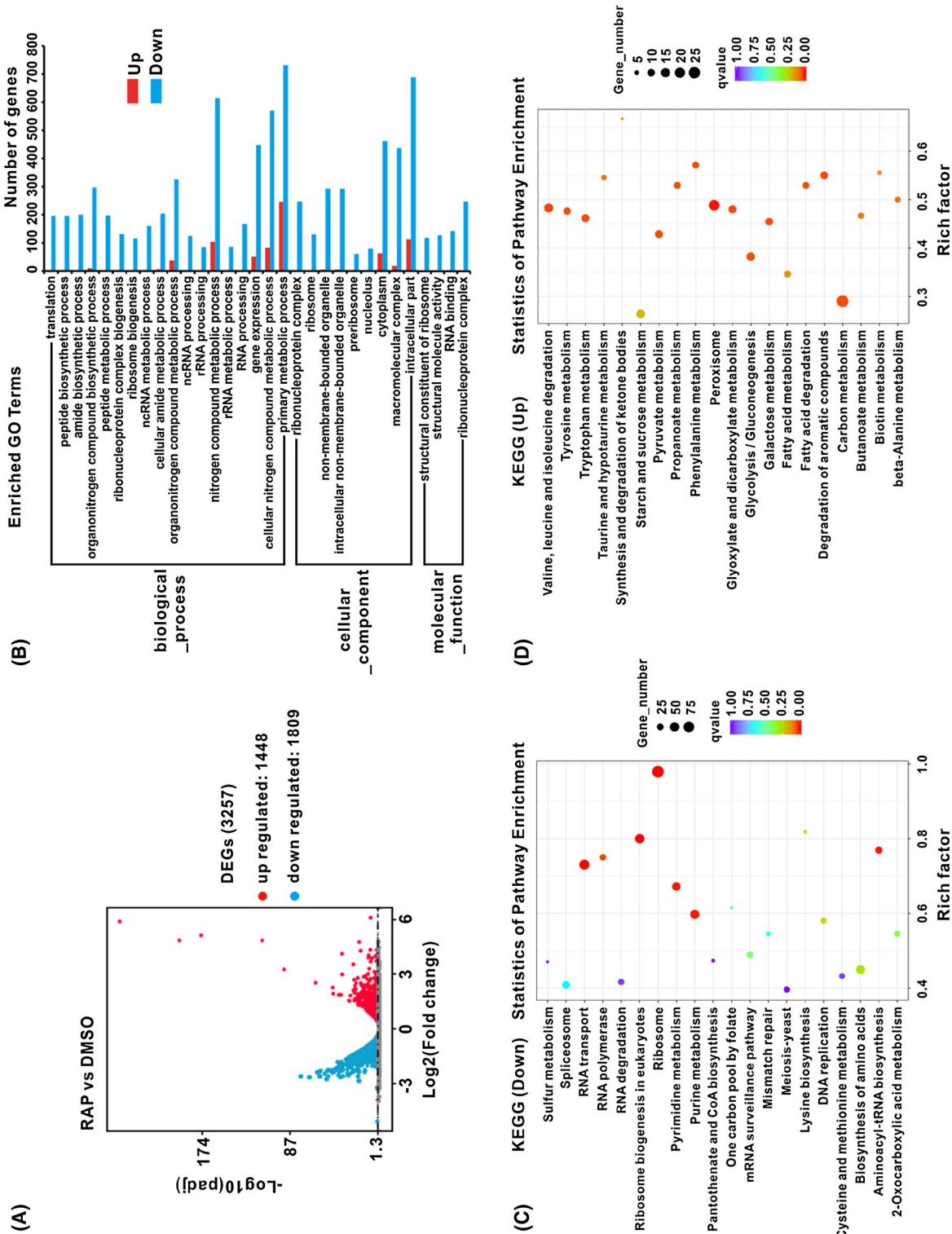


Fig. 5 RNA-Seq analysis of TOR function on growth of *Botrytis cinerea*. RNA sequencing was performed between rapamycin and DMSO treatment. Each treatment contained three biological replicates. (A) The number of DEGs. (B) Top 30 most significantly enriched GO terms. (C) Top 20 most enriched KEGG pathways for down-regulated DEGs. (D) Top 20 most enriched KEGG pathways for up-regulated DEGs.

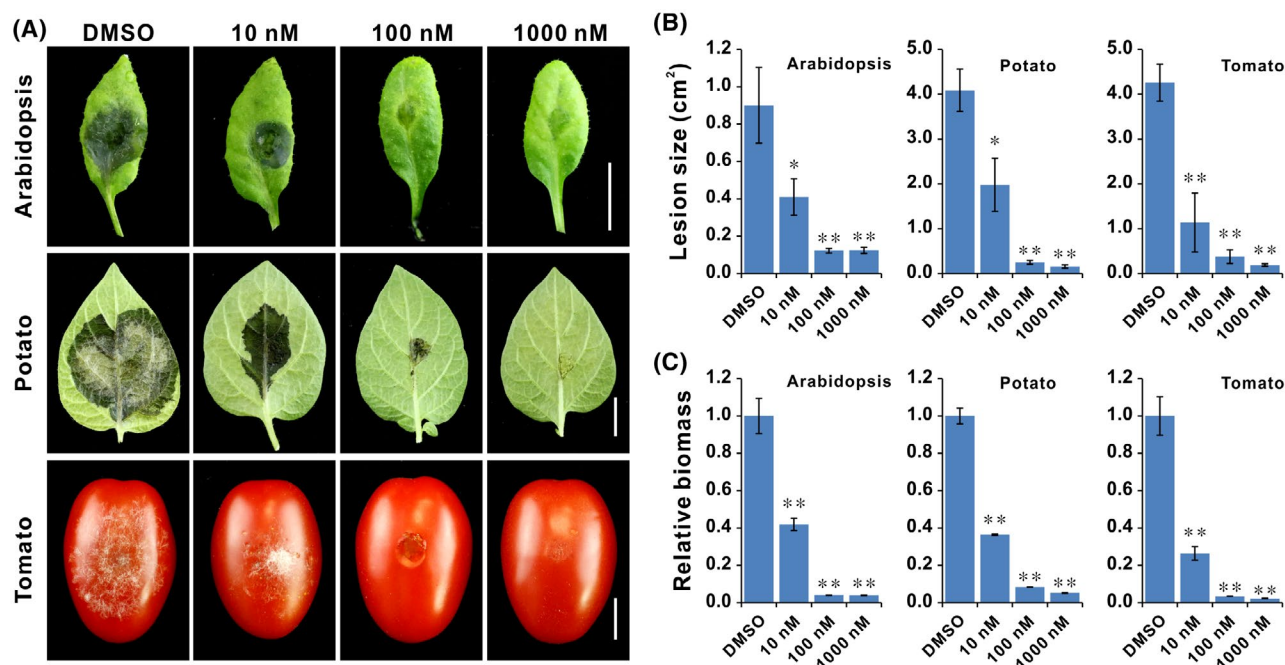


Fig. 6 TOR inhibition reduces infection by *Botrytis cinerea*. Leaves of *Arabidopsis* and potato, as well as fruits of cherry tomato, were infected by *B. cinerea* by dropping a spore suspension mixed with various doses of rapamycin. (A) Infected phenotype of *Arabidopsis* and potato leaves (3 days post-inoculation, dpi), and tomato fruits (7 dpi). (B, C) Lesion size and *B. cinerea* biomass were measured. Bar = 1 cm. Error bars indicate the SEM ($n \geq 20$). The experiments were repeated more than three times for each treatment. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

plants (Fig. 7E). These results suggest that plants expressing dsRNA targeting *BcTOR* could efficiently reduce susceptibility to *B. cinerea* by interfering with the expression of *BcTOR*.

The same constructs were used to generate transgenic tomato and a total of 27 independent transgenic lines were obtained (Fig. S4). Southern blotting results showed that among the selected lines, the BcRI1 and BcRI3 transgenic tomato lines contained two or more T-DNA insertions, while both BcRI2 transgenic lines had a single copy (Fig. S5A–C). To demonstrate the specificity of the *BcTOR* sRNA molecules generated in these transgenic lines, we carried out sRNA-Seq. The data showed that a large number of *BcTOR*-specific sRNA molecules were generated in these transgenic lines (Fig. 8A–C and Tables S5–S7). Corresponding to copy number, BcRI1 and BcRI3 lines had a higher abundance of sRNA molecules than BcRI2 lines. In addition, BcRI1 and BcRI3 lines also contained more species of *BcTOR*-specific sRNAs compared with BcRI2 lines or WT plants (Fig. 8A–C and Tables S5–S7). These results further confirm the presence of sRNA molecules derived from *BcTOR* RNAi constructs in transgenic tomato seedlings. Furthermore, pathogen infection experiments showed that, similar to the observation from the T1 generation, the T2 generation plants also displayed enhanced resistance against *B. cinerea*, suggesting that the trait is stable among generations (Figs 9A–E and S6A–D). Specifically, under normal growth conditions there was no difference between transgenic tomato plants expressing

dsRNA targeting *BcTOR* and WT tomato plants; however, dsRNA-expressing plants showed a distinct growth advantage after infection by *B. cinerea* compared with WT plants, where leaves showed serious decay (Fig. 9A). Additionally, consistent with *BcTOR*-specific sRNA levels, tomato fruits from BcRI1 and BcRI3 lines had smaller lesions and less pathogen biomass than BcRI2 lines or WT fruits, as well as a decline in transcript levels of *BcTOR* during infection (Fig. 9B–E). Taken together, these results further demonstrate that *BcTOR* dsRNA-expressing plants could constitutively produce *BcTOR*-specific sRNAs and effectively inhibit the expression of *BcTOR* to resist *B. cinerea*, suggesting that *BcTOR* is an effective target for genetic engineering in the control of grey mould.

DISCUSSION

To control grey mould disease, the traditional strategy is to apply fungicides, but fungicide residues derived from the widespread use of chemical pesticides have caused serious environmental pollution issues (Malhat *et al.*, 2015; Oliveira *et al.*, 2015; Tomenson and Matthews, 2009). Biofungicides for key targets of pathogens are a promising application for controlling diseases. On the other hand, genetic engineering methods also are extensively applied to improve resistance to plant pathogens for crop protection. For instance, HIGS has been proved to be an efficient tool to unravel

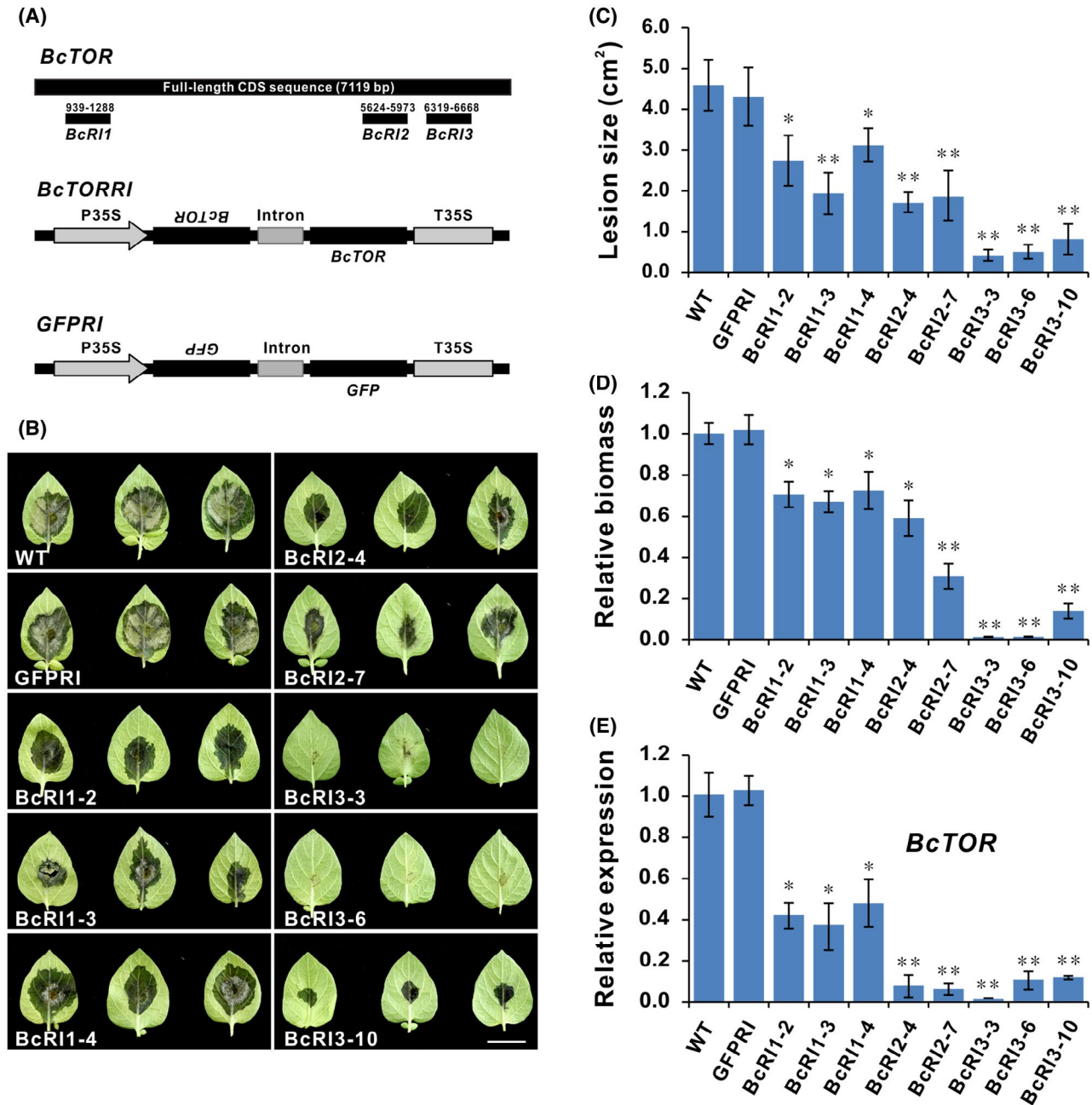


Fig. 7 Transgenic potato expressing *BcTOR* dsRNA shows enhanced resistance to *Botrytis cinerea*. (A) Schematic diagram of RNA interference vector targeting *BcTOR* and *GFP* (control). (B) Infection phenotype of *B. cinerea* on detached leaves of transgenic potato lines and wild-type (WT). Photographs were taken at 3 days post-inoculation. Scale bar represents 2 cm. (C, D) Infection lesion size and relative biomass of *B. cinerea*. Error bars indicate the SEM ($n \geq 20$). The experiments were repeated three times. (E) Relative expression level of *BcTOR* in *B. cinerea* during infection of transgenic and WT potato plants. Data are represented as means \pm SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

gene function as well as a promising approach to improve resistance to plant fungal pathogens by targeting key genes associated with growth and virulence (Cai *et al.*, 2018; Wang, Thomas *et al.*, 2017). However, selection of efficient targets is the key for HIGS.

TOR, a functionally and structurally conserved protein kinase, is a master regulator that controls cell growth by integrating

nutrient, energy and growth factors in all eukaryotic species (van Dam *et al.*, 2011; Takahara and Maeda, 2013; Wullschlegel *et al.*, 2006). TOR is extensively used as an excellent target in the control or therapy of diseases like Alzheimer's disease, Huntington's chorea and some cancers by using rapamycin or other active-site TOR inhibitors (Blenis, 2017; Ciuffreda *et al.*,

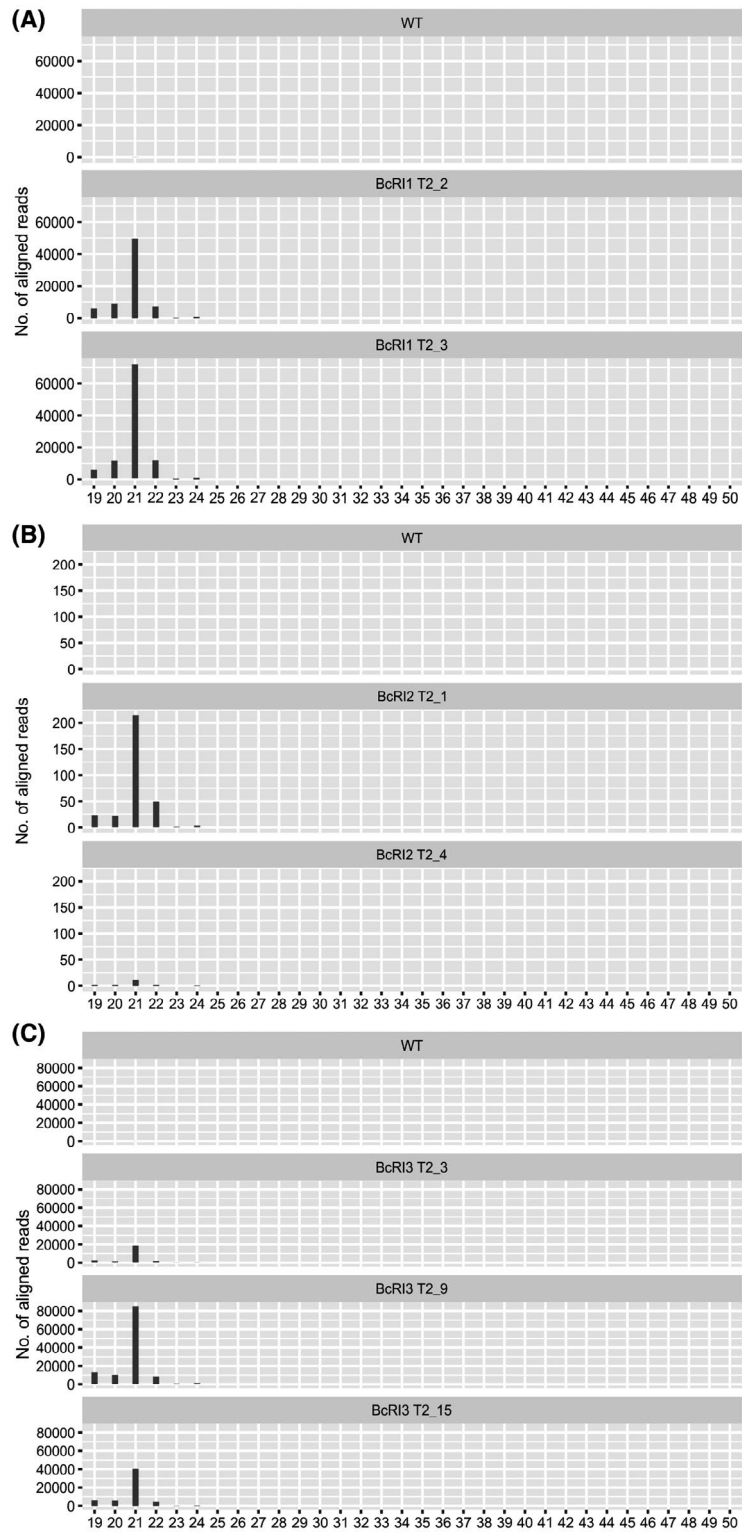


Fig. 8 The abundance of sRNAs targeting *BcTOR* in transgenic tomato lines expressing *BcTOR* dsRNA. Two-week-old transgenic and wild-type (WT) seedlings were harvested and used for small RNA (sRNA) sequencing. (A) sRNAs derived from BcRI1 construct lines. (B) sRNAs derived from BcRI2 construct lines. (C) sRNAs derived from BcRI3 construct lines.

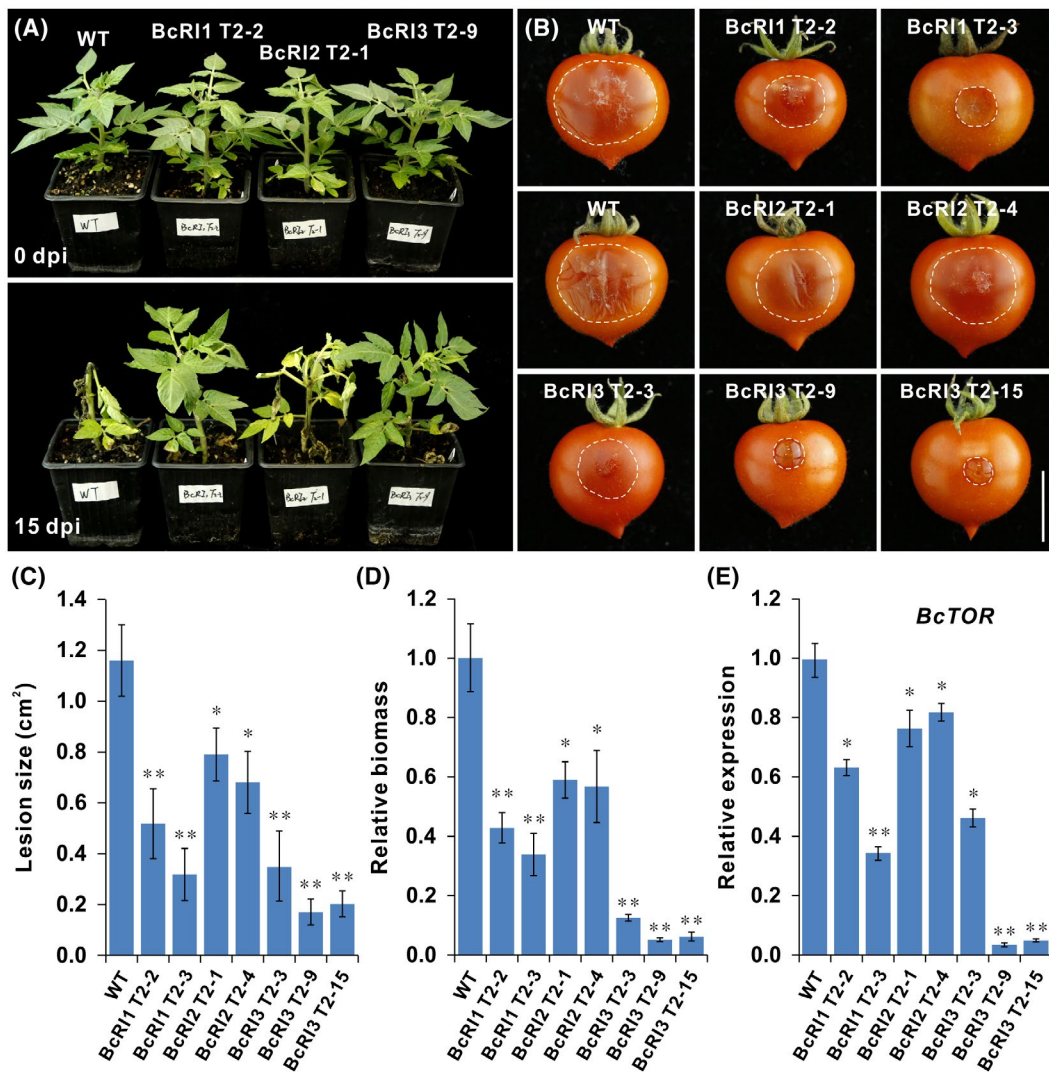


Fig. 9 Transgenic tomato expressing *BcTOR* dsRNA shows enhanced resistance to *Botrytis cinerea*. (A) Phenotype of *BcTOR* dsRNA-expressing tomato plants and wild-type (WT) before and after infection by *B. cinerea*. Scale bar represents 1 cm. (B) Infection phenotype of *B. cinerea* on fruits from transgenic tomato and WT plants. Photographs were taken at 3 days post-inoculation. Scale bar represents 1 cm. (C, D) Infection lesion size and relative biomass of *B. cinerea*. Error bars indicate the SEM ($n \geq 10$). The experiments were repeated three times. (E) Relative expression level of *BcTOR* in *B. cinerea* during infection of transgenic and WT tomato fruits. Data are represented as means \pm SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

2010; Easton and Houghton, 2006; Jung *et al.*, 2018; Santos *et al.*, 2011; Wang, Valera *et al.*, 2017), implying that TOR could also be a potential target in pathogens for disease control. To date, TOR signalling in phytopathogenic fungi such as *B. cinerea*, *M. circinelloides* and *Fusarium* spp. has been reported. TOR signalling was particularly well characterized in *F. graminearum* (Bastidas *et al.*, 2012; Lopez-Berges *et al.*, 2010; Melendez *et al.*, 2009; Teichert *et al.*, 2006; Yu *et al.*, 2014), suggesting that a conserved TOR pathway also exists in plant pathogenic fungi.

Genome scanning revealed the presence of conserved TOR signalling including conserved TOR protein and TOR components such as RAPTOR, LST8 of TORC1 and RICTOR of TORC2 in

B. cinerea (Fig. 1A–C and Table 1), as well as the FKBP12 homologue, suggesting there is conserved TOR signalling in *B. cinerea*. Previous studies showed that the *BcFKBP12* deletion relieved the growth inhibition of rapamycin, suggesting the role of *BcFKBP12* in associating the conserved inhibitory effect of rapamycin to TOR in *B. cinerea* (Gioti *et al.*, 2006). Based on the characteristics of this dependency, functional FKBP12 is usually used in studying TOR function in some rapamycin-insensitive species, especially in the model plant *Arabidopsis* (Mahfouz *et al.*, 2006; Ren *et al.*, 2012; Sormani *et al.*, 2007; Xiong *et al.*, 2017). For example, *Arabidopsis* plants depicted susceptibility to rapamycin by expressing *FKBP12* from yeast or human (Mahfouz *et al.*, 2006; Ren *et al.*, 2012; Sormani *et al.*, 2007; Xiong *et al.*, 2017). Consistent

with the previous studies, BcFKBP12 also restored the sensitivity of *Arabidopsis* plants to rapamycin (Fig. 2A–E). This result further confirmed the conserved function of BcFKBP12 in bridging the inhibitory effect of rapamycin to TOR.

The TOR signalling pathway plays critical roles in controlling cell growth and proliferation, and in modulating downstream transcription, translation, autophagy and metabolism processes in a variety of eukaryotes (Dobrenel *et al.*, 2016; Henriques *et al.*, 2014; Mahoney *et al.*, 2009; Rexin *et al.*, 2015; Thoreen *et al.*, 2012; Xiong and Sheen, 2015). Previous reports indicated that rapamycin suppresses growth of *B. cinerea*, but there is no detailed description of TOR function in these studies. Previously it has been reported that inhibition of TOR by rapamycin caused serious growth retardation, displaying twisted hyphal morphology, more branches and increased septation in *F. graminearum* (Yu *et al.*, 2014). In this study, similar results were observed in *B. cinerea* when treated with rapamycin (Figs 3A–C and S1), suggesting that TOR plays a crucial role in regulating vegetative growth in *B. cinerea*. For mycelial growth, the retarded growth caused by rapamycin was supported by RNA-Seq data that showed that many DEGs associated with ribosome, transcription and translation were down-regulated under TOR inhibition (Fig. 5B–D and Tables S2–S4). These data also reflect conserved functions of TOR signalling for ribosome biogenesis and assembly, transcription and translation (Takahara and Maeda, 2013; Wullschleger *et al.*, 2006). In addition, similar to previous reports in *F. graminearum*, TOR inhibition also led to severe suppression of conidiation of *B. cinerea* when concentrations of more than 10 nM rapamycin were used (Fig. 4A,B). This implies that restricting the spread of *B. cinerea* by inhibiting TOR could be a promising approach. TOR inhibition reduces pathogenicity in *F. graminearum* associated with regulating mycelial growth and virulence (Yu *et al.*, 2014), which offers an impressive example of TOR function on the virulence of plant fungal pathogens. Similarly, we observed reduction and even loss of infectivity in *B. cinerea* under TOR inhibition during infection (Fig. 6A–C), implying that BcTOR could be a potential target used to control *B. cinerea*.

HIGS is an effective strategy for crop protection and has also been proven to be effective in controlling necrotrophic fungal pathogens. For instance, *Arabidopsis* plants expressing dsRNA targeting *BcDCL1* and *BcDCL2* are more resistant to *B. cinerea* (Zhang *et al.*, 2016). Here, we generated transgenic potato and tomato plants expressing dsRNA targeting *BcTOR* based on the HIGS method. In general, the transgenic potato and tomato plants displayed enhanced, or near-complete resistance to *B. cinerea*, although there were some differences in resistance between transgenic lines (Figs 7B–E and 9A–E). sRNA-Seq showed that a large number of *BcTOR*-specific sRNA molecules were generated in these transgenic lines, and BcRI1 and BcRI3 lines had a higher abundance of sRNA molecules

than BcRI2 lines (Fig. 8A–C and Tables S5–S7). The data suggest that *BcTOR* dsRNA could be constitutively and efficiently processed into sRNA molecules (Fig. 8A–C and Tables S5–S7). These data also explain the difference in resistance between transgenic tomato lines (Figs 8A–C and 9A–E, and Tables S5–S7). In addition, the efficiency of dsRNA being processed into sRNAs may be responsible for differences among lines from different constructs. BcRI2 lines produced significantly fewer types of sRNA than BcRI1 and BcRI3 lines (Fig. 8A–C); an off-target effect may also be the cause of the differences among these constructs. Through aligning *BcTOR* RNAi fragments with the cDNA database of tomato or *B. cinerea*, we found no possible off-target genes in tomato plants, but in *B. cinerea* there were only two genes with more than 19 bases matched for each fragment (Table S9). Compared to BcRI2 lines, BcRI1 and BcRI3 contained more sRNA reads matching to non-target genes, but these multiple target sRNA reads only account for very few of the total sRNA reads in BcRI1 and BcRI3 lines (Tables S8 and S9). In summary, our results demonstrate that *BcTOR* dsRNA-expressing plants were able to effectively produce *BcTOR*-specific sRNAs against *B. cinerea*, reducing infection. This study suggests that TOR is a potential and promising target in the biocontrol of plant fungal pathogens.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

Botrytis cinerea strain B05.10 was incubated on PDA plates at 25 °C unless indicated otherwise. For the growth inhibition assay, 0.4 mm diameter agar plugs with fungal mycelia were placed on PDA plates with different concentrations of rapamycin (1, 10, 100, 1000 nM) or dimethylsulphoxide (DMSO, solvent control). The diameter of the colony was measured every day, and the hyphal tip growth and branching patterns were observed after 2 days' growth. For measurement of conidia production, spores were collected and counted after 10 days' cultivation. For the spore germination experiment, a spore suspension (1×10^6 spores/mL) was spread on PDA plates supplemented with rapamycin or DMSO for incubation, and spore germination was observed at different time points (2, 4 and 6 h).

In order to quantify the biomass of mycelia, potato dextrose broth (PDB) was used for liquid culture of mycelia. Five agar plugs with fungal mycelia were placed in a 100 mL triangular bottle with 50 mL PDB supplemented with the indicated concentrations of rapamycin or DMSO (solvent control), then the bottles were placed in an incubator at 25 °C. After 4 days' incubation, the mycelia were collected and freeze-dried to constant weight before measuring the biomass of mycelia. To detect the expression level of related genes, mycelia cultivated in PDB for 4 days were treated by adding the indicated concentrations of rapamycin or DMSO

(solvent control) for 12 h, and then the mycelia were collected for RNA extraction.

Plant material and growth conditions

The plant materials used in this work included *A. thaliana* ecotype Columbia (Col-0), *Solanum lycopersicum* 'Micro-Tom' and *Solanum tuberosum* 'Desirée'. All plants were grown in soil in an artificial climate culture room at 25/18 °C of day/night (except *Arabidopsis* plants were grown at 22 °C), 80% humidity and under a long-day photoperiod consisting of a 16 h light regime. In addition, cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) fruits purchased from the supermarket were also used in infection experiments.

Vector construction and plant transformation

Vector construction was based on a Gateway system according to previous reports (Earley *et al.*, 2006; Xiong *et al.*, 2017). Vector p35S-IN4-8GWN was used as the entry vector, and the plant binary vector KANA303, modified pEarleyGate303, was the destination vector (Xiong *et al.*, 2017). For RNAi vector construction, three different segments of 350-bp DNA sequences of *BcTOR* were selected, and the sense and antisense orientation fragments were then amplified with the primers (BcRI-L-Fu-F/BcRI-L-Fu-R) listed in Table S10a. The amplified products of antisense orientation fragments were then inserted into linearized p35S-IN4-8GWN (linearized by *NotI/SbfI*) to generate p35S-BcRI(L)-IN4-8GWN by seamless cloning using a In-Fusion HD Cloning Kit (Clontech, Palo Alto, CA, USA) following the user's manual. The sense fragments were amplified with primers (BcRI-R-Fu-F/BcRI-R-Fu-R) and linked into linearized p35S-BcRI(L)-IN4-8GWN (digested by *BbvCI/AscI*) to obtain p35S-BcRI(L)-IN4-BcRI(R)-8GWN by the seamless cloning method. Finally, RNAi fragments were cloned into the destination vector KANA303 using Gateway LR clonase (Invitrogen, Carlsbad, CA, USA).

The BcTOR-RNAi binary plasmid was transferred into *Agrobacterium* strain GV3101. Transgenic tomato was generated by *Agrobacterium*-mediated transformation following the protocols described by Fillatti *et al.* (1987), and transgenic potato created by *Agrobacterium*-mediated transformation according to the protocol described in Millam (2007). Transgenic plants were identified by PCR with primers of neomycin phosphotransferase II (*NPTII*) and *BcTOR* fragments (Table S10a). The positive transgenic plants were selected and used for subsequent experiments.

To create *BcFKBP12*-expressing *Arabidopsis*, the coding sequence of *BcFKBP12* (Bcin12g01360.1) was cloned in the plant binary vector KANA303 according to the experimental protocols described in previous reports (Xiong *et al.*, 2016). The resulting destination vectors were transferred into *Agrobacterium* strain GV3101 for plant transformation. The floral dipping method was employed for generating transgenic *Arabidopsis* (Zhang *et al.*, 2006). The transformation and screening of primary

transformants were performed according to Zhang *et al.* (2006), and T3 generation transgenic plants were used for subsequent experiments.

Botrytis cinerea infection

For inoculation of plants, conidia were harvested from sporulating colonies in sterile water with 0.1% Tween-20 and filtered with glass wool as described by Stefanato *et al.* (2009). For droplet inoculations, the concentration of spores was adjusted to 1×10^5 spores/mL and 5 μ L of spore suspension with the indicated dose of rapamycin or DMSO was dropped onto the surface of single detached rosette leaves of 5-week-old *Arabidopsis* plants or 45-day-old potato plants starting from tissue culture plantlets or mature red cherry tomato fruits from supermarket and mature red tomatoes (cv. Micro-Tom) harvested at 14 days after breaker stage from transgenic plants. Before inoculations, all detached leaves or fruits were surface cleaned or sterilized with 75% ethanol. The inoculated leaves or fruits were kept under a transparent plastic film cover to maintain high humidity and kept at 25 °C in the dark. Infection symptoms were observed and photographed at 3 days post-incubation (dpi). For the incubation of intact tomato plants, the same density of spore suspension was applied for spray inoculations of 45-day-old intact tomato plants. Plants were kept prior to and during infection under sealed hoods at high humidity, and infection symptoms were observed and photographed at 15 dpi. The lesion area was measured using ImageJ software (<http://imagej.nih.gov/ij/>) from digitally computed images of leaves or tomato fruits.

DNA extraction and DNA quantification

To quantify *B. cinerea* growth by real-time quantitative PCR (qPCR), 1 cm² of leaf tissue or 1 cm³ of fruit tissue around the infected lesion area was collected at 3 dpi, and then ground into powder in liquid nitrogen. Total DNA was isolated using a plant genomic DNA extraction kit (Biotেকে, Beijing, China) following the manufacturer's user manual. To estimate the amount of fungal DNA in inoculated samples, purified DNAs were used for qPCR as described by Zhang *et al.* (2013). The primers listed in Table S10b were used.

Expression profiling sequencing and analysis

RNA-Seq was carried out by Novogene Bioinformatics Technology Co. Ltd (Beijing, China). Hyphae of *B. cinerea* were grown for 4 days in PDB medium at 25 °C and then treated by adding 100 nM rapamycin or DMSO (as solvent control). For each treatment, three independent biological replicates were performed. After incubation for 12 h, the mycelia were collected and total RNA of *B. cinerea* mycelium was isolated using a RNAPrep Pure Plant Kit (Tiangen, Beijing, China). RNA integrity was assessed using the RNA Nano 6000

Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following manufacturer's recommendations, the library preparations were sequenced on an Illumina HiSeq platform and 125 bp/150 bp paired-end reads were generated. The clean reads were aligned to the reference *B. cinerea* genome using TopHat2 software (Kim *et al.*, 2013). Transcript abundance was also normalized by transforming the data to reads per kilobase of exon model per million mapped reads (FPKM) method. Cufflinks and Cuffdiff were used to assemble the mapped reads and identify DEGs, respectively (Trapnell *et al.*, 2012, 2013), and genes with an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed. All the DEGs were annotated in the *B. cinerea* genome database website (http://fungi.ensembl.org/Botrytis_cinerea/Info/Index?db=core). Gene ontology enrichment (corrected *P*-value < 0.05) of the DEGs was performed using GO sequencing software (Young *et al.*, 2010). The enrichment of DEGs in KEGG pathways (corrected *P*-value < 0.05) was obtained using KOBAS software (Kanehisa *et al.*, 2008; Mao *et al.*, 2005). RNA-Seq data was validated by real-time qRT-PCR for the 20 DEGs showed on Fig. S7 and the corresponding primers are listed in Table S10c.

RNA isolation and qRT-PCR analysis

Total RNA was isolated using the RNAPrep Pure Plant Kit (TianGen, Beijing, China, DP441) and reverse transcription was performed using the PrimeScript RT Reagent Kit (Takara, Otsu, Japan) following the manufacturer's instructions. Real-time PCR was performed on a Bio-Rad CFX96 System (Bio-Rad, Hercules, CA, USA) using the TB Green Premix Ex Taq (Takara, Otsu, Japan).

DNA gel blotting

The genomic DNA samples were isolated from 2-week-old T2 generation seedlings of transgenic tomato lines. DNA gel blotting was performed using the DIG-High prime DNA labelling and detection starter kit II (Roche, Mannheim, Germany) following the protocol provided with the kit. Genomic DNA was digested with the restriction enzyme *EcoRI* for DNA Southern blot analysis and about 10 µg of each was loaded on a 1% agarose gel. The probe location was a selected interference fragment of BcTOR, amplified and sequenced from corresponding plasmid with primers.

Small RNA sequencing

Total RNA extracted from 2-week-old transgenic and WT seedlings was used for sRNA sequencing. sRNA libraries were constructed using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Inc., Ipswich, MA, USA) according to the manufacturer's instructions. Briefly, 1 µg of total RNA was ligated to a 3' adapter and a 5' adapter using Ligation Enzyme Mix. The resulting samples were reverse-transcribed using Superscript II

reverse transcriptase. Amplification was executed for the PCR products. All steps were performed according to the manufacturer's protocols. sRNA libraries were analysed for quality control and the average size of inserts was approximately 140–150 bp. The sequencing library was then sequenced on a HiSeq platform (Illumina) by Shanghai Personal Biotechnology Cp. Ltd (Shanghai, China). The quality information of raw data in FASTQ format was calculated and the raw data were filtered using the Personalbio self-developed script. Clean data were obtained by removing adapter and low-quality sequence. Filter Clean Reads from 18 to 36 nt in length and perform deduplication to obtain Unique Reads for subsequent analysis. The unique reads were aligned using BLAST with the corresponding target fragment of *BcTOR*. The reads count value of the sRNA was counted based on the number of sequences aligned to the corresponding target fragment of *BcTOR*.

Statistics

All experiments were repeated and yielded reproducible results. The most representative data are shown in this paper. Data are presented as means ± standard error of the mean, unless stated otherwise. Paired or unpaired, two-tailed Student's *t*-tests were used to compare group differences. *P*-values < 0.05 were considered significant.

ACKNOWLEDGEMENTS

We thank Guozheng Qin (Chinese Academy of Sciences, Beijing, China) for kindly providing *B. cinerea* strain B05.10. This work was supported by the following grants: the National Natural Science Foundation of China (nos. 31801913, 31972469, 31672206 and 31801271), the China Postdoctoral Science Foundation (nos. 2017M622958 and 2018M633320), the Project of Chongqing Science and Technology Commission (nos. cstc2016jcyjA0822, cstc2019jcyj-msxmX0127 and cstckjcxljrc15), Chengdu Agricultural Science and Technology Center local financial special fund project (NASC2019T113), the Fundamental Research Funds for the Central Institutes and the Chinese Academy of Agricultural Sciences (19-001-09).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Baretic, D. and Williams, R.L. (2014) The structural basis for mTOR function. *Semin. Cell Dev. Biol.* **36**, 91–101.
- Bastidas, R.J., Shertz, C.A., Lee, S.C., Heitman, J. and Cardenas, M.E. (2012) Rapamycin exerts antifungal activity *in vitro* and *in vivo* against *Mucor circinelloides* via FKBP12-dependent inhibition of Tor. *Eukaryot Cell*, **11**, 270–281.

- Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J. (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–1326.
- Benjamin, D., Colombi, M., Moroni, C. and Hall, M.N. (2011) Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat. Rev. Drug Discov.* **10**, 868–880.
- Blenis, J. (2017) TOR, the gateway to cellular metabolism, cell growth, and disease. *Cell*, **171**, 10–13.
- Cai, Q., He, B., Kogel, K.H. and Jin, H. (2018) Cross-kingdom RNA trafficking and environmental RNAi-nature's blueprint for modern crop protection strategies. *Curr. Opin. Microbiol.* **46**, 58–64.
- Cheng, W., Song, X.S., Li, H.P., Cao, L.H., Sun, K., Qiu, X.L., Xu, Y.B., Yang, P., Huang, T., Zhang, J.B., Qu, B. and Liao, Y.C. (2015) Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to Fusarium head blight and seedling blight in wheat. *Plant Biotechnol. J.* **13**, 1335–1345.
- Chiu, M.I., Katz, H. and Berlin, V. (1994) RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Natl. Acad. Sci. USA*, **91**, 12574–12578.
- Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.M., Simon, A. and Viaud, M. (2007) *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphagous pathogen. *FEMS Microbiol. Lett.* **277**, 1–10.
- Ciuffreda, L., Di Sanza, C., Incani, U.C. and Milella, M. (2010) The mTOR pathway: a new target in cancer therapy. *Curr. Cancer Drug Tar.* **10**, 484–495.
- van Dam, T.J., Zwartkruis, F.J., Bos, J.L. and Snel, B. (2011) Evolution of the TOR pathway. *J. Mol. Evol.* **73**, 209–220.
- Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G.D. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **13**, 414–430.
- Dobrenel, T., Caldana, C., Hanson, J., Robaglia, C., Vincentz, M., Veit, B. and Meyer, C. (2016) TOR signaling and nutrient sensing. *Annu. Rev. Plant Biol.* **67**, 261–285.
- Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.
- Easton, J.B. and Houghton, P.J. (2006) mTOR and cancer therapy. *Oncogene*, **25**, 6436–6446.
- Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D. and Shokat, K.M. (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol.* **7**, e38.
- Fillatti, J.A.J., Kiser, J., Rose, R. and Comai, L. (1987) Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. *Nat. Biotechnol.* **5**, 726–730.
- Fruman, D.A. and Rommel, C. (2014) PI3K and cancer: lessons, challenges and opportunities. *Nat. Rev. Drug Discov.* **13**, 140–156.
- Ghag, S.B., Shekhawat, U.K. and Ganapathi, T.R. (2014) Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* **12**, 541–553.
- Gioti, A., Simon, A., Le Pecheur, P., Giraud, C., Pradier, J.M., Viaud, M. and Levis, C. (2006) Expression profiling of *Botrytis cinerea* genes identifies three patterns of up-regulation in planta and an FKBP12 protein affecting pathogenicity. *J. Mol. Biol.* **358**, 372–386.
- Govindarajulu, M., Epstein, L., Wroblewski, T. and Michelmore, R.W. (2015) Host-induced gene silencing inhibits the biotrophic pathogen causing downy mildew of lettuce. *Plant Biotechnol. J.* **13**, 875–883.
- Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell*, **110**, 177–189.
- Heitman, J., Movva, N.R. and Hall, M.N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253**, 905–909.
- Helliwell, S.B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. and Hall, M.N. (1994) TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Cell. Biol.* **5**, 105–118.
- Henriques, R., Bogre, L., Horvath, B. and Magyar, Z. (2014) Balancing act: matching growth with environment by the TOR signalling pathway. *J. Exp. Bot.* **65**, 2691–2701.
- Jung, S., Gamez-Diaz, L., Proietti, M. and Grimbacher, B. (2018) "Immune TOR-opathies," a novel disease entity in clinical immunology. *Front. Immunol.* **9**, 966.
- Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T. and Yamanishi, Y. (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* **36**, D480–484.
- Kim, D., Perlea, G., Trapnell, C., Pimentel, H., Kelley, R. and Salzberg, S.L. (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **14**, R36.
- Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J. and Kogel, K.H. (2013) Host-induced gene silencing of cytochrome P450 lanosterol C14alpha-demethylase-encoding genes confers strong resistance to Fusarium species. *Proc. Natl. Acad. Sci. USA*, **110**, 19324–19329.
- Koltin, Y., Faucette, L., Bergsma, D.J., Levy, M.A., Cafferkey, R., Koser, P.L., Johnson, R.K. and Livi, G.P. (1991) Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl cis-trans isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* **11**, 1718–1723.
- Loewith, R. and Hall, M.N. (2011) Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics*, **189**, 1177–1201.
- Loewith, R., Jacinto, E., Wullschlegel, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M.N. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell*, **10**, 457–468.
- Lopez-Berges, M.S., Rispaill, N., Prados-Rosales, R.C. and Di Pietro, A. (2010) A nitrogen response pathway regulates virulence functions in *Fusarium oxysporum* via the protein kinase TOR and the bZIP protein MeaB. *Plant Cell*, **22**, 2459–2475.
- Madeira da Silva, L. and Beverley, S.M. (2010) Expansion of the target of rapamycin (TOR) kinase family and function in *Leishmania* shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity. *Proc. Natl. Acad. Sci. USA*, **107**, 11965–11970.
- Mahfouz, M.M., Kim, S., Delauney, A.J. and Verma, D.P. (2006) Arabidopsis TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals. *Plant Cell*, **18**, 477–490.
- Mahoney, S.J., Dempsey, J.M. and Blenis, J. (2009) Cell signaling in protein synthesis ribosome biogenesis and translation initiation and elongation. *Prog. Mol. Biol. Transl.* **90**, 53–107.
- Malhat, F.M., Haggag, M.N., Loutfy, N.M., Osman, M.A. and Ahmed, M.T. (2015) Residues of organochlorine and synthetic pyrethroid pesticides in honey, an indicator of ambient environment, a pilot study. *Chemosphere*, **120**, 457–461.
- Mao, X., Cai, T., Olyarchuk, J.G. and Wei, L. (2005) Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics*, **21**, 3787–3793.
- Melendez, H.G., Billon-Grand, G., Fevre, M. and Mey, G. (2009) Role of the *Botrytis cinerea* FKBP12 ortholog in pathogenic development and in sulfur regulation. *Fungal Genet. Biol.* **46**, 308–320.
- Millam, S. (2007) Potato (*Solanum tuberosum* L.). In *Agrobacterium Protocols* (K. Wang ed.), pp. 25–35. Totowa, NJ: Humana Press.
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumblehn, J. and Schweizer, P. (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell*, **22**, 3130–3141.

- Nunes, C.C. and Dean, R.A. (2012) Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. *Mol. Plant Pathol.* **13**, 519–529.
- Oliveira, B.R., Penetra, A., Cardoso, V.V., Benoliel, M.J., Barreto Crespo, M.T., Samson, R.A. and Pereira, V.J. (2015) Biodegradation of pesticides using fungi species found in the aquatic environment. *Environ. Sci. Pollut. R.* **22**, 11781–11791.
- Pedras, M.S., Hossain, S. and Snitynsky, R.B. (2011) Detoxification of cruciferous phytoalexins in *Botrytis cinerea*: spontaneous dimerization of a camalexin metabolite. *Phytochemistry*, **72**, 199–206.
- Ren, M., Venglat, P., Qiu, S., Feng, L., Cao, Y., Wang, E., Xiang, D., Wang, J., Alexander, D., Chalivendra, S., Logan, D., Mattoo, A., Selvaraj, G. and Datla, R. (2012) Target of rapamycin signaling regulates metabolism, growth, and life span in *Arabidopsis*. *Plant Cell*, **24**, 4850–4874.
- Rexin, D., Meyer, C., Robaglia, C. and Veit, B. (2015) TOR signalling in plants. *J. Biochem.* **470**, 1–14.
- Robaglia, C., Thomas, M. and Meyer, C. (2012) Sensing nutrient and energy status by SnRK1 and TOR kinases. *Curr. Opin. Plant Biol.* **15**, 301–307.
- Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S.H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell*, **78**, 35–43.
- Santos, R.X., Correia, S.C., Cardoso, S., Carvalho, C., Santos, M.S. and Moreira, P.I. (2011) Effects of rapamycin and TOR on aging and memory: implications for Alzheimer's disease. *J. Neurochem.* **117**, 927–936.
- Sehgal, S.N., Baker, H. and Vézina, C. (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J. Antibiot.*, **28**, 727–732.
- Sauer, E., Imseug, S., Maier, T. and Hall, M.N. (2013) Conserved sequence motifs and the structure of the mTOR kinase domain. *Biochem. Soc.* **41**, 889–895.
- Schmelzle, T. and Hall, M.N. (2000) TOR, a central controller of cell growth. *Cell*, **103**, 253–262.
- Song, Y. and Thomma, B. (2018) Host-induced gene silencing compromises *Verticillium* wilt in tomato and *Arabidopsis*. *Mol. Plant Pathol.* **19**, 77–89.
- Sormani, R., Yao, L., Menand, B., Ennar, N., Lecampion, C., Meyer, C. and Robaglia, C. (2007) *Saccharomyces cerevisiae* FKBP12 binds *Arabidopsis thaliana* TOR and its expression in plants leads to rapamycin susceptibility. *BMC Plant Biol.* **7**, 26.
- Stefanato, F.L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C.G., Metraux, J.P. and Schoonbeek, H.J. (2009) The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. *Plant J.* **58**, 499–510.
- Takahara, T. and Maeda, T. (2013) Evolutionarily conserved regulation of TOR signalling. *J. Biochem.* **154**, 1–10.
- Teichert, S., Wottawa, M., Schonig, B. and Tudzynski, B. (2006) Role of the *Fusarium fujikuroi* TOR kinase in nitrogen regulation and secondary metabolism. *Eukaryot. Cell*, **5**, 1807–1819.
- Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S. and Sabatini, D.M. (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature*, **485**, 109–113.
- Tomenson, J.A. and Matthews, G.A. (2009) Causes and types of health effects during the use of crop protection chemicals: data from a survey of over 6,300 smallholder applicators in 24 different countries. *Int. Arch. Occ. Env. Health.* **82**, 935–949.
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L. and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578.
- Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L. and Pachter, L. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* **31**, 46–53.
- Vezina, C., Kudelski, A. and Sehgal, S.N. (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot.* **28**, 721–726.
- Villa-Rojas, R., Sosa-Morales, M.E., Lopez-Malo, A. and Tang, J. (2012) Thermal inactivation of *Botrytis cinerea* conidia in synthetic medium and strawberry puree. *Int. J. Food Microbiol.* **155**, 269–272.
- Wang, X. and Proud, C.G. (2009) Nutrient control of TORC1, a cell-cycle regulator. *Trends Cell Biol.* **19**, 260–267.
- Wang, M., Weiberg, A., Lin, F.M., Thomma, B.P., Huang, H.D. and Jin, H. (2016) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants*, **2**, 16151.
- Wang, M., Thomas, N. and Jin, H. (2017) Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre- and post-harvest plant protection. *Curr. Opin. Plant Biol.* **38**, 133–141.
- Wang, Z., Valera, J.C., Zhao, X., Chen, Q. and Silvio Gutkind, J. (2017) mTOR co-targeting strategies for head and neck cancer therapy. *Cancer Metast. Rev.* **36**, 491–502.
- Williamson, B., Tudzynski, B., Tudzynski, P. and van Kan, J.A. (2007) *Botrytis cinerea*: the cause of grey mould disease. *Mol. Plant Pathol.* **8**, 561–580.
- Wullschlegel, S., Loewith, R. and Hall, M.N. (2006) TOR signaling in growth and metabolism. *Cell*, **124**, 471–484.
- Xiong, Y. and Sheen, J. (2015) Novel links in the plant TOR kinase signaling network. *Curr. Opin. Plant Biol.* **28**, 83–91.
- Xiong, F., Dong, P., Liu, M., Xie, G., Wang, K., Zhuo, F., Feng, L., Yang, L., Li, Z. and Ren, M. (2016) Tomato FK506 binding protein 12KD (FKBP12) mediates the interaction between rapamycin and target of rapamycin (TOR). *Front. Plant Sci.* **7**, 1746.
- Xiong, F., Zhang, R., Meng, Z., Deng, K., Que, Y., Zhuo, F., Feng, L., Guo, S., Datla, R. and Ren, M. (2017) Brassinosteroid Insensitive 2 (BIN2) acts as a downstream effector of the Target of Rapamycin (TOR) signaling pathway to regulate photoautotrophic growth in *Arabidopsis*. *New Phytol.* **213**, 233–249.
- Xu, J., Wang, X., Li, Y., Zeng, J., Wang, G., Deng, C. and Guo, W. (2018) Host-induced gene silencing of a regulator of G protein signalling gene (VdRGS1) confers resistance to *Verticillium* wilt in cotton. *Plant Biotechnol. J.* **16**, 1629–1643.
- Yang, H., Rudge, D.G., Koos, J.D., Vaidialingam, B., Yang, H.J. and Pavletich, N.P. (2013) mTOR kinase structure, mechanism and regulation. *Nature*, **497**, 217–223.
- Young, M.D., Wakefield, M.J., Smyth, G.K. and Oshlack, A. (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* **11**, R14.
- Yu, F., Gu, Q., Yun, Y., Yin, Y., Xu, J.R., Shim, W.B. and Ma, Z. (2014) The TOR signaling pathway regulates vegetative development and virulence in *Fusarium graminearum*. *New Phytol.* **203**, 219–232.
- Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W. and Chua, N.H. (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* **1**, 641–646.
- Zhang, Y., Butelli, E., De Stefano, R., Schoonbeek, H.J., Magusin, A., Pagliarini, C., Wellner, N., Hill, L., Orzaez, D., Granell, A., Jones, J.D. and Martin, C. (2013) Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. *Curr. Biol.* **23**, 1094–1100.
- Zhang, T., Jin, Y., Zhao, J.H., Gao, F., Zhou, B.J., Fang, Y.Y. and Guo, H.S. (2016) Host-induced gene silencing of the target gene in fungal cells confers effective resistance to the cotton wilt disease pathogen *Verticillium dahliae*. *Mol. Plant*, **9**, 939–942.
- Zhu, X., Qi, T., Yang, Q., He, F., Tan, C., Ma, W., Voegelé, R.T., Kang, Z. and Guo, J. (2017) Host-induced gene silencing of the MAPKK Gene PsFUZ7 confers stable resistance to wheat stripe rust. *Plant Physiol.* **175**, 1853–1863.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 Biomass of *B. cinerea* under rapamycin and DMSO treatment in a liquid culture condition.

Fig. S2 Phenotype of tomato fruits infected by *B. cinerea* under TOR inhibition at 15 dpi.

Fig. S3 Identification of transgenic potato by PCR with primers of *neomycin phosphotransferase II (NPTII)* and *BcTOR* fragments.

Fig. S4 Identification of transgenic tomato by PCR with primers of *neomycin phosphotransferase II (NPTII)* and *BcTOR* fragments.

Fig. S5 DNA gel blotting analysis of tomato transgenic lines.

Fig. S6 Phenotype of *BcTOR* dsRNA expressing tomato in T1 generation.

Fig. S7 Validation of RNA-seq data by real-time PCR.

Table S1 DEGs between rapamycin and DMSO treatment

Table S2 Enriched GO terms

Table S3 Enriched KEGG pathway (down)

Table S4 Enriched KEGG pathway (up)

Table S5 Abundance statistics of sRNA target to BcTOR

Table S6 sRNA molecule sequence information

Table S7 sRNA blast information

Table S8 Potential off-target genes in *B. cinerea*

Table S9 sRNAs matching to non-targeting genes

Table S10 Primers used in this study