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SHORT COMMUNICATION

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TOR signaling downregulation increases resistance to the cereal killer *Fusarium graminearum*

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ABSTRACT

TOR is the master regulator of growth and development that senses energy availability. Biotic stress perturbs metabolic and energy homeostasis, making TOR a good candidate to participate in the plant response. *Fusarium graminearum* (*Fusarium*) produces important losses in many crops all over the world. To date, the role of TOR in *Fusarium* infection has remained unexplored. Here, we show that the resistance to the pathogen increases in different *Arabidopsis* mutants impaired in TOR complex or in wild-type plants treated with a TOR inhibitor. We conclude that TOR signaling is involved in plant defense against *Fusarium*.

The protein kinase target of rapamycin (TOR) is a master regulator that controls growth and development integrating external and internal signals (nutrients availability, hormones, etc).[8,6] It is well documented that TOR signaling regulates many important biological processes such as protein translation, but it is still unclear its role in environmental stresses. In plants, TOR associates to RAPTOR (Regulatory-associated protein of TOR) and LST8 (Lethal with Sec 13) proteins to form a multiprotein complex denominated TOR COMPLEX 1. One sequence coding for a TOR protein and two for both RAPTOR (*RAPTOR3G* and *RAPTOR5G* genes, At3g08850 and At5g01770 respectively) and LST8 (*LST8.1* and *LST8.2* genes, At3g18140 and At2g22040 respectively) can be retrieved from the Arabidopsis genome. Despite the occurrence of two genes coding for each TOR-partner, only *RAPTOR3G* and *LST8.1* are expressed in high levels.[6] Conversely, *AtRAPTOR5G* is transcribed in very low levels and expression of *AtLST8.2* has not been detected.[1,4,11]

TOR is essential to maintain the energy and metabolic homeostasis of the cells, which is extremely affected under biotic stresses. *Fusarium* sp. is one of the most important genera of fungal plant pathogens, causing devastating diseases in numerous economically important crops. *Fusarium graminearum* (*Fusarium*), which causes the disease Fusarium Head Blight (FHB) of wheat, barley and other cereals, is the most widespread species.[15] FHB produces cereal grain yield and quality reduction, and also contamination with mycotoxins, which are dangerous for animals and even for humans. Given that, the need for investigations about signaling pathways involved in the defense response to this dangerous fungus is evident in order to develop new crop protection strategies.

In this work, we focus on the study of the role of TOR complex in the *Arabidopsis thaliana*-*Fusarium* interaction. It was described that *Fusarium* is able to infect the model plant *Arabidopsis*.[14,3,13] First, we analyzed the effect of *Fusarium* on *Arabidopsis* wild-type (WT) and different TOR-complex

mutant plants, TOR conditional mutant (estradiol-inducible RNA interference, herein called *tor-es*),[16] *raptor3g*, *lst8.1* and *lst8.2*. Detached-leaf infection bioassays were performed as described previously[3] using cut leaves from rosette of 3-weeks-old plants. A virulent isolate of *Fusarium* was used to inoculate the leaves and disease severity was assessed during 7 days post inoculation (dpi). As shown in Fig. 1A-D, *Fusarium* infection caused important disease symptoms in WT plants, being susceptible to the fungus. However, a resistant reaction was observed in *tor-es* and *raptor3g* mutant lines, in comparison to WT plants. This is evidenced through scarcely fungus growth, presence of fewer plant dead cells (Fig. 1B), decreasing disease severity (between 5–10%, Fig. 1C) and reduction of the lesion size (Fig. 1D). The mutant line impaired in *LST8.1* expression was more resistant to the fungal infection than the WT but more susceptible than *tor-es* and *raptor3g* (30% lower disease severity) (Fig. 1A-C).

However, the lesion sizes in the *lst8.1* mutants were smaller than those presented in the WT leaves, indicating a reduction of the severity of the symptoms (Fig. 1D). On the other hand, *lst8.2* mutant presented a similar infection level than WT plants, being the disease severity almost 90% (Fig. 1A-D). We also performed an infection assay in whole plant of the *raptor3g* mutant to corroborate the results in detached leaves. As expected, the mutant was more resistant to *Fusarium* infection, with a value of whole plant decay below 20% (Fig. 1E-G).

To further confirm the results, we conducted a pharmacological assay with the ATP-competitive TOR kinase inhibitor PP242.[17] *Arabidopsis* WT plants were treated with PP242 and the symptoms and degree of infection by *Fusarium* were analyzed. The inhibitor treated-WT plants were less infected than the control (Fig. 2). This result demonstrates that the downregulation of TOR signaling confers more resistance/tolerance to *Fusarium* infection.
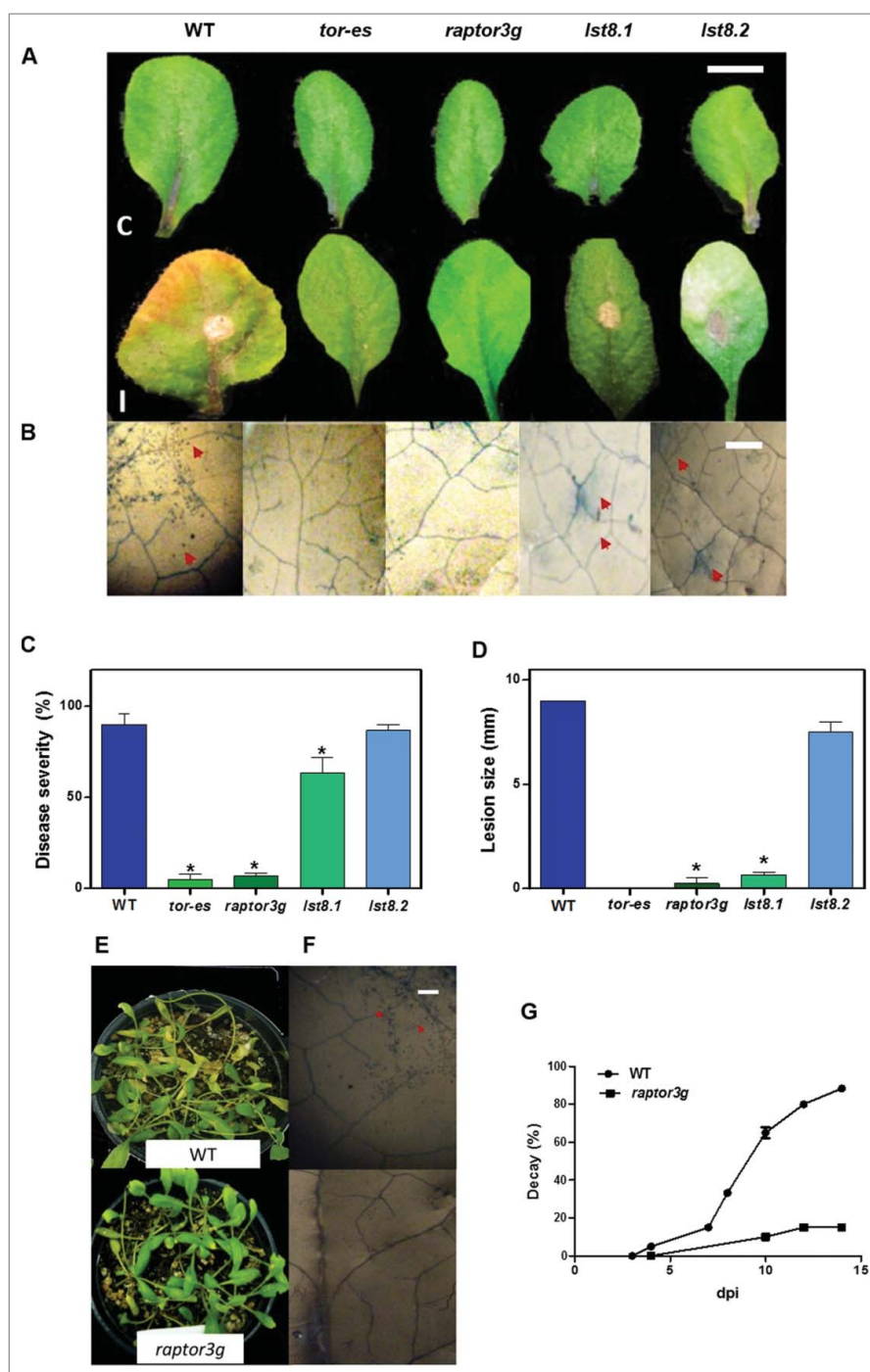
Figure 1. *Arabidopsis thaliana* TOR complex mutants are more resistant to *Fusarium graminearum* infection. (A) Disease reaction of *Arabidopsis* TOR complex mutants. Lesion development in WT and mutants leaves (3-weeks-old seedlings) at 7 dpi with *Fusarium* (5 μl of 1–5 $\times$ 10^5 conidia/ml suspension) (I) compared with their respective control (C). For induction of tor-RNAi of *tor-es* mutant, 10 μM of β-estradiol was added 24 h previous to infection. Representative images are shown. Scale bar = 2 cm. (B) *Fusarium* mycelial growth and plant cell death. The same leaves of (A) were stained with trypan blue reagent and visualized under stereoscope (20x). Representative images are shown. The red arrowheads indicate cell death and infectious hyphae on inoculated leaves. Scale bars = 200 μm. (C) Assessment of resistance to *Fusarium* infection of *Arabidopsis* WT and TOR complex mutants. Quantification of development disease in leaves of WT and TOR mutants plants at 7 dpi with *Fusarium*. Standard error bars (SE) are shown. Significant statistically differences (Tukey's test) are indicated with asterisks (P < 0.05). (D) Lesion size measurement. Expanding lesions were measured on excised leaves at 7 dpi with *Fusarium*. SE are indicated. Significant statistically differences (Tukey's test) are indicated with asterisks (p < 0.05). (E) *Fusarium* infection on *Arabidopsis* whole plants. Symptoms in *Arabidopsis* WT and *raptor3g* mutant after infection with 20 ml of *Fusarium* suspension (5 μl of 1–5 $\times$ 10^5 conidia/ml suspension) at 14 dpi. Representative images are shown. (F) *Fusarium* mycelial growth and plant cell death. The same leaves of (E) was stained with trypan blue reagent and visualized under stereoscope (20x). Representative images are shown. Scale bar = 200 μm. (G) Whole plant decay of (E) was measured each day of the disease assay. SE are shown.

In summary, we demonstrated that TOR signaling may be involved in Arabidopsis-*Fusarium* interaction, using both TOR complex-proteins mutants and a second generation TOR inhibitor (PP242). The *tor-es* and *raptor3g* mutants were more resistant genotypes to *Fusarium* infection than *Arabidopsis* WT plants. One explanation of the results obtained with the TOR complex mutants is that plants behave as constitutively "primed" against pathogen. Priming is defined as an induced state whereby
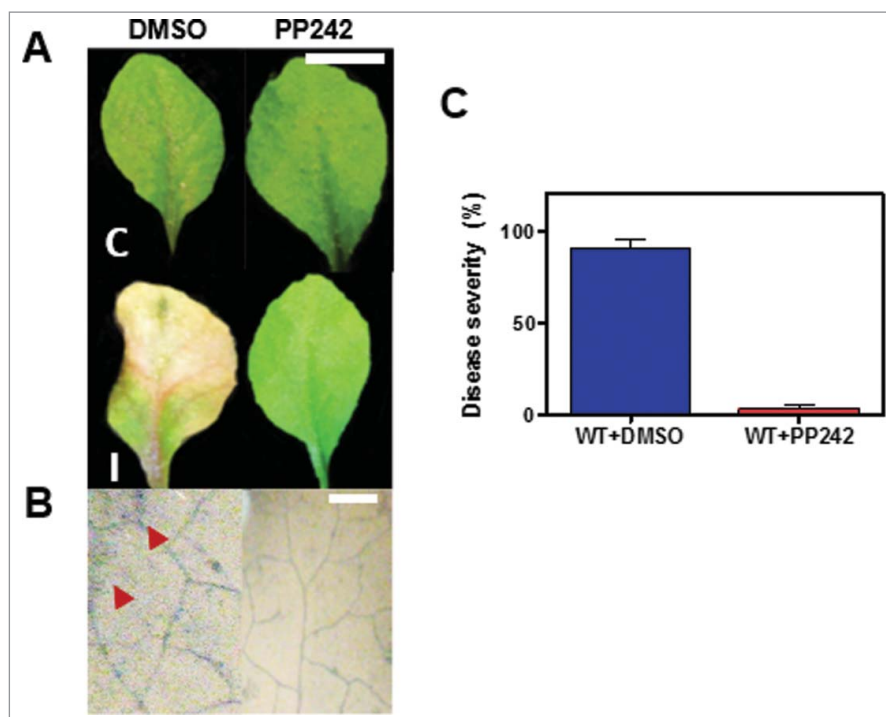
Figure 2. *Arabidopsis* WT plants with pharmacological inhibition of TOR signaling are more resistant to *Fusarium* infection. (A) Disease reaction of WT plants impaired in TOR signaling. Lesion development in *Arabidopsis* WT or WT leaves treated with the TOR inhibitor PP242 (2 μM) at 7 dpi with *Fusarium* (5 μl of 1–5 $\times$ 10^5 conidia/ml suspension) (I) compared with their respective control (C). Scale bar = 2 cm. Representative images are shown. (B) *Fusarium* mycelial growth and plant cell death. The same leaves of (A) were stained with trypan blue reagent and visualized under stereoscope (20x). The red arrowheads indicate cell death and infectious hyphae on inoculated leaves. Scale bars = 200 μm. (C) Assessment of resistance to *Fusarium* infection in pharmacological TOR-inhibited *Arabidopsis* WT leaves. Quantification of disease development of WT or PP242-treated WT leaves (2 μM) at 7 dpi with *Fusarium*. Standard error bars (SE) are indicated.

a plant responds more rapidly and/or more efficiently to a stress.[2] In accordance with the mutant behavior against *Fusarium* infection, *Arabidopsis* WT plants treated with the TOR kinase inhibitor, PP242, were almost totally resistant to the fungus attack. Previously, it was reported that TOR signaling has a role for the potyvirus infection in *Arabidopsis* and that the inhibition of TOR activity prevents the disease.[12] During the writing of this manuscript, Meteignier et al. (2017) and De Vleesschauwer et al. (2018)[10,5] have described that TOR mutants could enhance resistance to different pathogens in *Arabidopsis* and in rice respectively. Our results are in agreement with both reports, contributing with an extremely important example to the idea that the role of TOR in the plant response to pathogens could be general.

Finally, our data demonstrate that TOR complex is involved in the *Arabidopsis* response against the fungus *Fusarium* which causes numerous losses of cereal crops. These results contribute to the knowledge of the mechanisms that are involved in plant defense responses, which are indispensable for biotechnology purposes. The fact that affecting RAPTOR and LST8.1 protein levels also increases the resistance to *Fusarium* broadens the possibility of manipulating the TOR pathway, without modifying TOR expression, and thus decreasing negative side effects.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Columbia-0 ecotype) was used as genetic background. TOR conditional mutant seeds (estradiol-inducible RNAi, *tor-es*) were a kind gift from Dr. Xiong.[16] The other mutants of the TOR-Complex: *raptor3g* (SALK_022096C[9]), *lst8.1* and *lst8.2* (SALK_002459 and SALK_018605C respectively[11]) were obtained from SALK collection (http://signal.salk.edu/). Screening for the T-DNA knockout mutants was performed by PCR analysis (Table S1). *tor-es* mutant plants were treated with 10 μM of β-estradiol (E8875; SIGMA-Aldrich, USA) for induction of *tor*-RNAi 24 h previous to infection assays. The treatment with estradiol did not affect *Fusarium* infection (Fig. S1).

Plants were grown in pots containing soil mixture (3:1:1 mixture of peat moss-based mix:vermiculite:perlite) under a 16:8 h (light-dark) photoperiod at 22°C.

Disease assays

Leaves from 3-weeks-old plants were used in detached leaf infection bioassay system as described previously.[3] Petioles of detached leaves were embedded into sterile water agar (0.7%) with the leaf blade not touching the agar surface. A *Fusarium graminearum* native isolate from INBIOTEC-FIBA collection was used for the infection experiments. For leaf inoculation assays, 5-μl drops of conidial suspension (1–5 $\times$ 10^5 conidia/ml) or water (control) were put onto the adaxial leaf surface. The plates were incubated under a 16:8-h light-dark cycle at 22°C (100% RH), and disease severity was assessed 24, 48, 72 h and 7 d post inoculation (dpi). Disease progression in the inoculated leaves was estimated by an average disease rating (0–4) as follow: the fraction of inoculated leaves exhibiting chlorosis

covering <25% (category 1), 25 to 50% (category 2), 50 to 75% (category 3), and >75% (category 4) of leaf area was determined for each genotype. A minimum of 30 leaves of each genotype was analyzed for each experiment. Disease severity (as percentage) was calculated for each treatment according to the following formula, $DS = [(\Sigma\ nRi)/(NRmax)] \times 100$, where n is the number of leaves with each disease rating (Ri), N is the total number of leaves of each line, and R max is the maximum disease rating (corresponding to 4).

For the infection assays of whole plants (4-weeks old), 20 ml of *Fusarium* suspension were sprayed ($1–5 \times 10^5$ conidia/ml) and disease decay was determined during 14 d according Guo and Stotz.[7]

Microscopical analysis of infection

Leaves were transferred to chloral hydrate solution (2.5 g/ml) for clearing, and the infection process became evident by boiling infected leaves for 4 min in trypan blue solution containing equal volumes of 1 mg/ml aqueous trypan blue, lactic acid, glycerol and water. Microscopic inspection was carried out at 7 dpi using a Nikon stereoscopy (Stereoscopic Zoom Microscope SMZ 800).

TOR inhibitor treatment

Leaves from 3-weeks-old seedlings were treated or not with 2 μM PP242 (stock solution was dissolved in DMSO; P0037 SIGMA-Aldrich) in agar plates and inoculated with *Fusarium* as pointed above. DMSO (0.1%) was added in plates without the inhibitor as controls. Pictures were taken at 7 dpi.

Statistical analysis

The GraphPad Prism Version 5.01 program was used for statistical analysis. One-way ANOVA was performed and the differences between groups were tested using Tukey's Multiple Comparison Test with p-value< 0.05. Three independent experiments were conducted for all the assays performed in this study.

Abbreviations

dpi	days after inoculation
FHB	Fusarium Head Blight
LST8	Lethal with Sec 13
RAPTOR	Regulatory-associated protein of TOR
TOR	Target of Rapamycin
WT	Wild Type.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Author contributions

GMN conceived the project. GMN and FC designed and supervised the experiments and NA performed them. GMN wrote the manuscript with the help and advice of GLS.

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