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SHORT COMMUNICATION

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Plasmopara viticola effector PvRXLR159 suppresses immune responses in *Nicotiana benthamiana*

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ABSTRACT

Plasmopara viticola, the causal oomycete of grapevine downy mildew disease, secrets a series of RXLR effectors to manipulate host immunity. In this study, we characterized the role of a RXLR effector of *P. viticola*, PvRXLR159, in plant–microbe interaction. Transcription of *PvRXLR159* in *P. viticola* was induced in the early stage of infection in grapevine (*Vitis vinifera* 'Thomson Seedless'). Further results revealed that PvRXLR159 contains a functional signal peptide and its C terminus was essential to inhibit cell death by elicitors, INF1 and BAX, in *Nicotiana benthamiana*. Transient expression of PvRXLR159 suppressed *N. benthamiana* resistance to a pathogenic oomycete, *Phytophthora capsici*. Taken together, we propose that PvRXLR159 is induced and secreted by *P. viticola* to suppress host resistance.

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Introduction

Plants have evolved a two-layered innate immune system against pathogen infection. The first layer is termed as pattern-triggered immunity (PTI), which is triggered by the recognition via pattern recognition receptors (PRRs) of conserved molecules from microbes called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), such as bacterial flagellin peptides and an elicitin INF1 from oomycete. As a ubiquitous defense response in plants, PTI generally includes a series of rapid responses such as a burst of reactive oxygen species (ROS), callose deposition, activation of mitogen-activated protein kinases (MAPKs) and defense genes expression.[1–4] On the other hand, most pathogens like bacteria, fungi, and oomycetes secrete effectors to suppress PTI for successful infection. As a counter measure, resistant plants recognize these effectors via resistance (R) proteins triggering the second layer of plant defense termed as effector-triggered immunity (ETI), limiting the proliferation of pathogens.[5]

RXLR effectors (for Arg, any amino acid, Leu and Arg) and CRNs effectors (crinkling and necrosis induced protein) are two important classes of effectors in pathogenic oomycetes, and both of them contain modular structures, including N-terminal signal peptide responsible for transporting effector proteins into cells followed by some conserved motifs, like RXLR, QXLR, LFLAK and CHXC (X stands for any amino acid).[5–10] So far, more than 500 putative RXLR and CRN effectors have been predicted by bioinformatics analysis.[6,11–13] A major function of those effectors is to suppress plant resistance.[14] It is known that C-terminal domains of RXLR effectors is generally important for their function in manipulating plant immunity.[15]

Plasmopara viticola is an obligate biotrophic oomycete that causes devastating downy mildew disease of grapevine, resulting in tremendous economic loss in the grape and wine industry worldwide.[16] So far, more than 100 candidate RXLR effectors have been predicted from *P. viticola*.[17–22] A comprehensive functional study of 83 PvRXLR effectors has found that 52 of them including PvRXLR159 completely suppressed cell death induced by elicitors, INF1 and BAX, when expressed in *Nicotiana benthamiana*, suggesting that they are potential suppressors in manipulating plant immunity.[23] Our recent studies revealed that PvRXLR131 suppresses host resistance by targeting BRI1 kinase inhibitor 1.[24] In this study, we characterized the role of PvRXLR159 in plant–microbe interaction.

Materials and methods

Plant materials and growth conditions

The grapevine (*V. vinifera* 'Thompson Seedless') and *N. benthamiana* used in this study were grown in a green house at 25°C with a photoperiod of 14 h light/10 h darkness.

Vector construction

The oligonucleotides used for plasmid construction in this study are documented in the Supporting Information (Table S1). The signal peptide sequence of PvRXLR159 was predicted using the

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online site SingalP4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/). *Eco*RI and *Xho*I restriction enzymes were subsequently used to insert the signal peptide sequences into the pSUC2 vector. For the deletion experiment of suppressing cell death in *N. benthamiana*, different truncated sequences of *PvRXLR159* were inserted into vector pGR106 using the *Sal*I and *Cla*I restriction enzymes. For *Phytophothora capsici* infection assay, the sequence encoding *PvRXLR159* without signal peptide was integrated into the vector PBI121-EGFP using *Bam*HI and *Spe*I restriction enzymes.

Functional verification of signal peptide

Functional validation of the predicted signal peptide of PvRXLR159 was conducted with signal sequence trap (SST) assay.[25,26] The pSUC2 vector used in this experiment contains the coding sequence of a truncated invertase, SUC2, without the initial methionine and signal peptide. The sequences of signal peptides of Avr1b and PvRXLR159, and the first 25 amino acids of Mg87 were fused in frame to the invertase gene in the pSUC2 vector, respectively. The recombinant plasmids were then transformed into invertase secretion-deficient yeast strain YTK12 by lithium acetate-mediated transformation,[27] and then the suspension was inoculated on CMD-W medium to screen the positive transformants. The positive yeast colonies were transferred to YPRAA medium and 0.1% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution to verify the secretion of invertase.

Agrobacterium tumefacien-*mediated transient expression in* N. benthamiana

Plasmids were transformed into *A. tumefacien* strain GV3101 via electroporation, and then incubated in LB medium supplemented with appropriate antibiotics for 2 days.[28] The cells were harvested by centrifugation at 4000 × *g* for 5 min and resuspended in 10 mM $MgCl_2$ to adjust OD_{600} to 0.4 for cell death assay and 0.6 for other experiments. Finally, the suspension was infiltrated into *N. benthamiana* leaves from the abaxial side using needleless syringes for expression.[29]

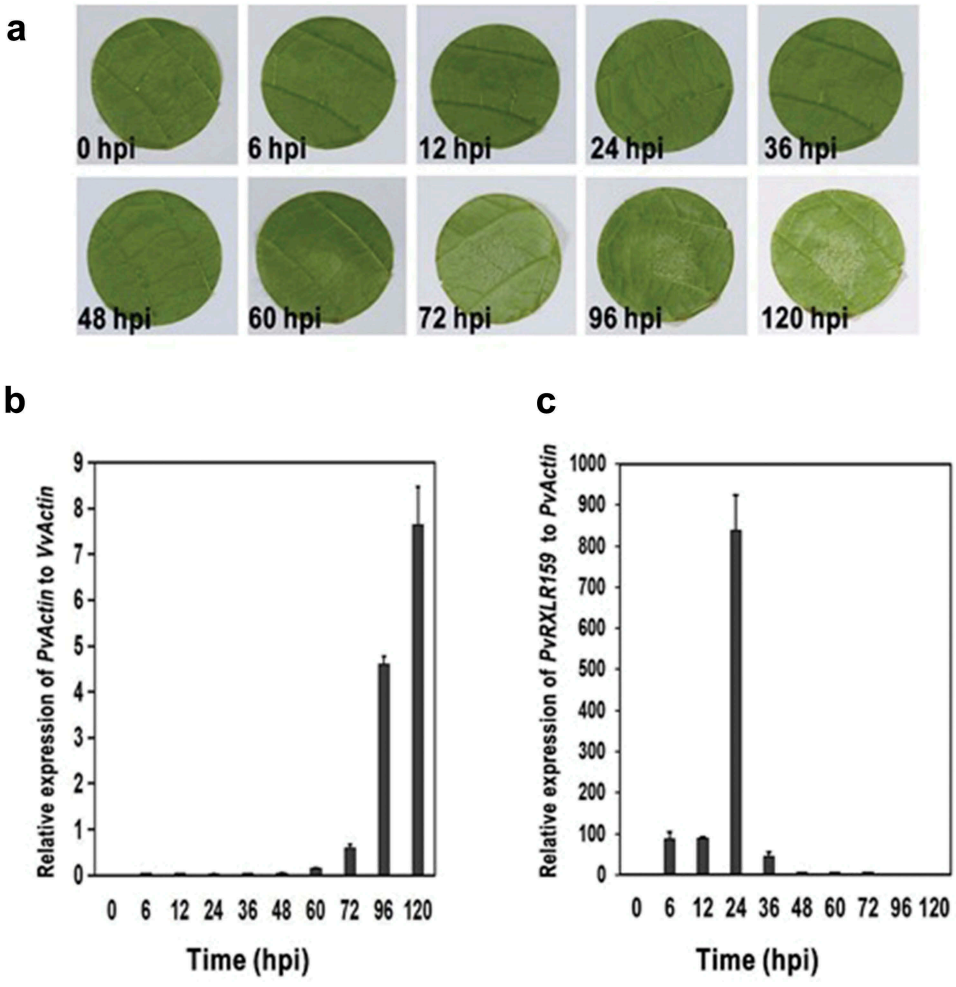

Figure 1. *PvRXLR159* is induced in the early stage of infection.

A, Representative photos of grapevine leaf discs inoculated with *P. viticola*. B, The growth of *P. viticola* in grapevine. The growth of *P. viticola* was monitored by qPCR and evaluated as the relative quantity of *PvActin* (*P. viticola* Actin) to *VvActin* (*V. vinifera* Actin). C, Transcription of *PvRXLR159* in *P. viticola* during infection. Transcription levels of *PvRXLR159* were monitored by qPCR and evaluated as the relative quantity of *PvRXLR159* to *PvActin*. Error bars represent the standard errors from three biological replicates. hpi, hours post-inoculation.

Pathogen infection assay

Infection of *P. viticola* 'JL-7-2' was performed according to previous studies.[20,24] The susceptible grapevine (*V. vinifera* 'Thompson Seedless') leaf discs were inoculated with 30 μL spore suspension with a final concentration of 1×10^5 spores/mL on the abaxial side, and then incubated in an incubator (18°C, 11 h light/13 h dark, 66% relative humidity). Phenotypic monitoring and sampling for RNA extraction were performed at indicated times. For *P. capsici* infection assay, *P. capsici* was grown on oatmeal agar medium (30 g oat, 20 g agar, 1 L water) for 4–5 days in the dark at 25°C. Agar discs with a diameter of 7 mm harboring *P. capsici* mycelium were put on detached leaves of *N. benthamiana* for infection.

DNA and RNA extraction and real-time quantitative PCR analysis

DNA and RNA from plant tissues were extracted using CTAB method[30] and a plant RNA kit (Omega, Norcross, GA, USA), respectively. cDNA was synthesized using One-Step gDNA Removal and cDNA Synthesis Super Mix kit (Trans Gen Biotech). qPCR was performed using SYBR Green Fast qPCR Mix and StepOne Real-Time PCR system (Bio-rad, Hercules, CA, USA). Primers for qPCR were listed in Table S2.

Protein extraction and western blot analysis

Protein of *N. benthamiana* was extracted according to our previous studies.[23] The plant tissues were ground into powder with liquid nitrogen, and then subjected to protein extraction in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM DTT, 1 mM PMSF, 1× protease inhibitor cocktail, 5 μM MG132). Equal amounts of protein were separated by SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk and then incubated with anti-GFP as primary antibody for 1 h, followed by incubation with secondary antibody for 1 h. The signal was detected by Super Signal West Femto ECL kit (Thermo Scientific, Rockford, IL, USA).

Results

PvRXLR159 transcription is induced in the early stage of infection

We investigated the expression pattern of *PvRXLR159* in *P. viticola* during infection in grapevine by qRT-PCR. The growth of *P. viticola* on leaf discs was slow during the period from 0 to 60 h after inoculation, and then increased dramatically at later growth stages (Figure 1(a,b)). The abundance of *PvRXLR159* transcripts peaked at around 24-h post-inoculation (hpi) and then dropped rapidly (Figure 1(c)). These results indicate that *PvRXLR159* is induced in the early stage of infection, which may contribute to the growth of *P. viticola* in grapevine. Note that we could not detect transcripts of *P. viticola* at 0 hpi due to the very low abundance of *P. viticola* in leaf discs.

PvRXLR159 is an effector containing functional signal peptide

Previous studies have shown that many RXLR effectors have N-terminal signal peptides composed of hydrophobic

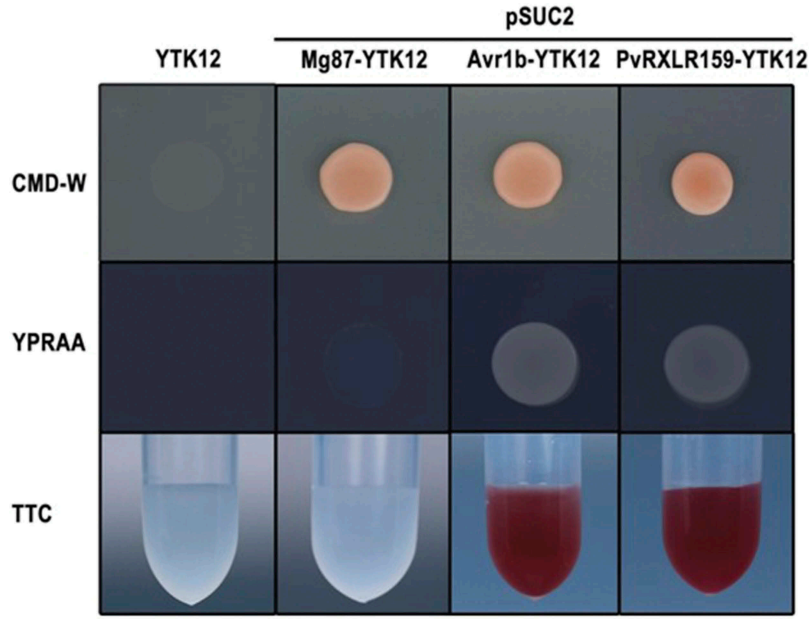

Figure 2. PvRXLR159 is an effector containing functional signal peptide.

Functional validation of the signal peptide of PvRXLR159 was carried out by using signal sequence trap assay. The signal peptide sequences of *PvRXLR159* and *Avr1b* as a positive control, and the sequence of the first 25 amino acids of *Mg87* as a negative control were fused in frame to the invertase gene in the pSUC2 vector, which were transformed into yeast YTK12 strain. CMD-W medium were used to screen positive transformants. YPRAA medium and TTC reducing assay were used to test invertase secretion. Only clones having a functional signal peptide can grow on YPRAA medium and reduce TTC to red formazan.

amino acids and are secreted by oomycetes.[6,31,32] We used singalP4.0 to predict the signal peptide region of PvRXLR159 and found that the first 21 amino acids at the N terminus of PvRXLR159 are a potential signal peptide region with a high probability of 0.76. Hence, we performed the SST assay to validate whether the N-terminal signal peptide of PvRXLR159 has secretory function.[26,33] The signal peptide sequences of *Avr1b* as a positive control and *PvRXLR159*, and the sequence coding the first 25 amino acids of *Mg87* as a negative control were fused in frame to the invertase gene in the pSUC2 vector, which were transformed into the invertase secretion-deficient yeast strain YTK12.[34,35] YTK12 carrying pSUC2-Avr1b and pSUC2-PvRXLR159 were able to grow on YPRAA medium with raffinose as the sole carbon source and reduce the dye TTC to the red-colored insoluble TTF,

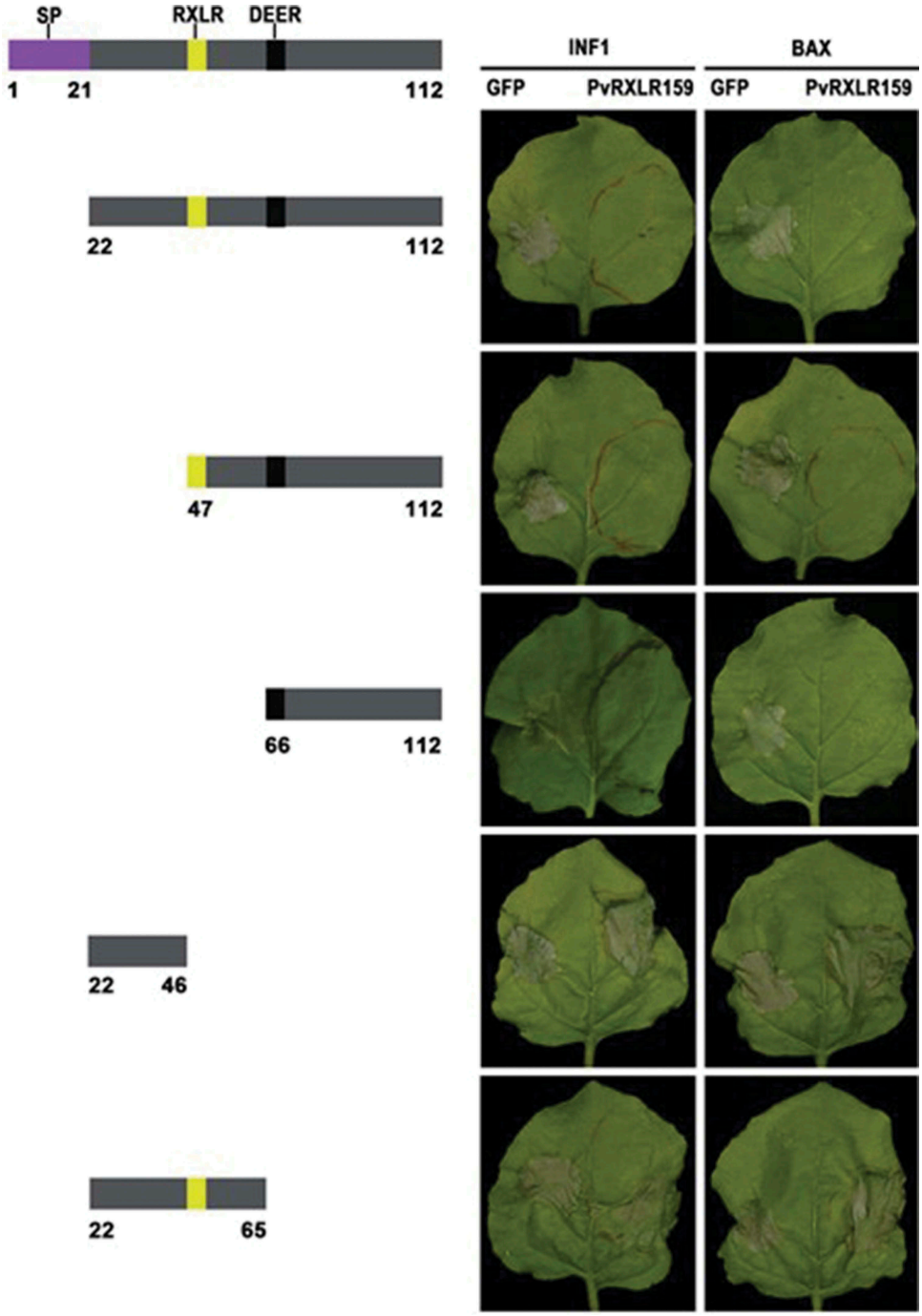

Figure 3. C terminus of PvRXLR159 is essential for its function.

Deletion mutants of PvRXLR159 were expressed in *N. benthamiana* leaves for 24 h, followed by expression of the elicitors INF1 and BAX to induce cell death. Left panel, a schematic diagram of the different deletion constructs. Right panel, typical symptoms of *N. benthamiana* taken at 5 days after expression of INF1 and BAX. The experiments were repeated at least three times with similar results.

whereas the YTK12 strain without transformation or carrying pSUC2-Mg87 was not (Figure 2), indicating that signal peptide of PvRXLR159 is functional.

C terminus of PvRXLR159 is essential for its function

Our previous studies have shown that PvRXLR159 suppresses INF1-and BAX-triggered cell death when transiently expressed in *N. benthamiana*.[23] Based on this phenotype, we constructed several deletion mutants of PvRXLR159 to investigate which domain of PvRXLR159 is important for its function. The deletion mutants containing 66 to 112 C-terminal sequence of PvRXLR159 retained the ability to suppress INF1-and BAX-triggered cell death, whereas the mutants having N-terminal sequences did not (Figure 3). These results indicate that the C terminus of PvRXLR159 is essential for blocking cell death triggered by INF1 and BAX.

PvRXLR159 suppresses *N. benthamiana* resistance against *phytophthora capsici*

Since genetic modification of *P. viticola* and grapevine are both challenging, we investigated whether PvRXLR159 manipulates plant immunity against pathogenic oomycete using the *N. benthamiana-P. capsici* pathosystem instead. As shown in Figure 4, expression of PvRXLR159 in *N. benthamiana* significantly increased the lesion size induced by *P. capsici* infection, indicating that infiltration of PvRXLR159 in *N. benthamiana* enhances susceptibility to *P. capsici*.

Conclusions

In the present study, we functionally characterized PvRXLR159 with the following findings: 1) transcription of *PvRXLR159* in *P. viticola* is induced during infection; 2) PvRXLR159 has a functional signal peptide; 3) C terminus is essential for its inhibition of INF1- and BAX-induced cell death in *N. benthamiana*; 4) PvRXLR159 suppresses *N. benthamiana* resistance against *P. capsici*. In addition, expression of PvRXLR159 also suppressed Arabidopsis resistance to a pathogenic bacterium, *Pseudomonas syringae pv. tomato* DC3000 (data not shown), suggesting that the suppression of host resistance by PvRXLR159 is a conserved phenotype in plants. Based on these results, we propose that PvRXLR159 is induced and secreted by *P. viticola* to suppress host resistance. Since PvRXLR159 is localized in both nucleus and cytoplasm when expressed in plants,[23] it may have different targets in these compartments. Research is undergoing to identify the targets of

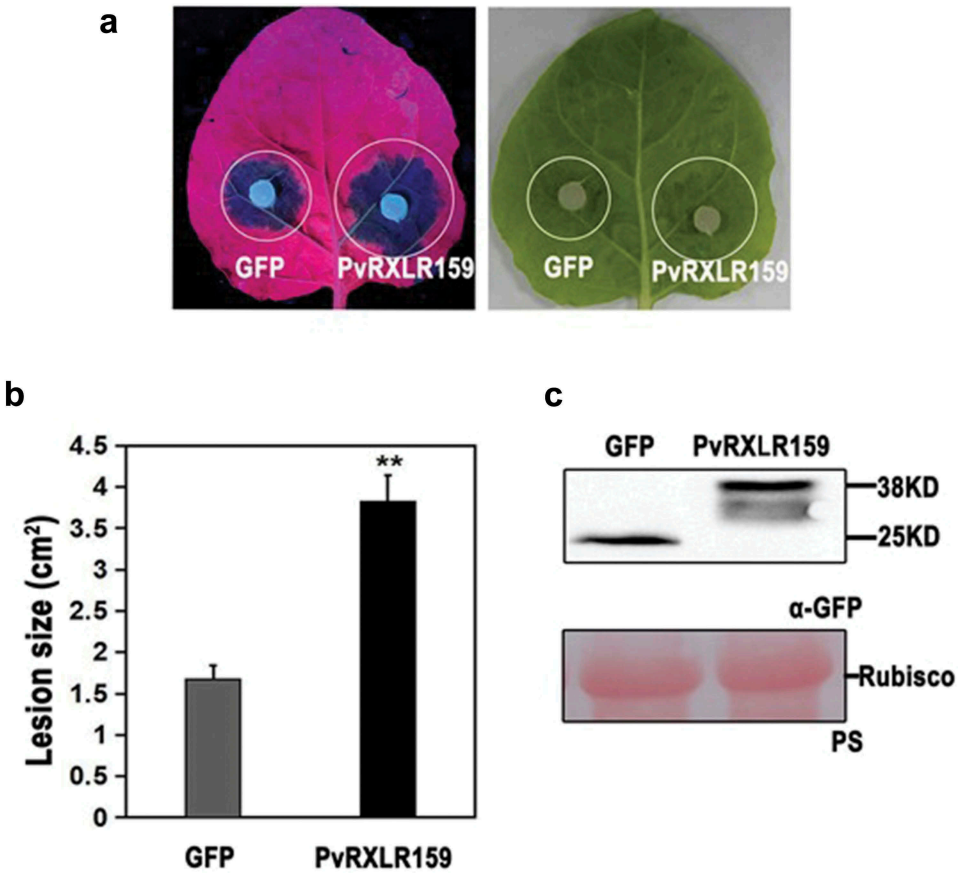

Figure 4. PvRXLR159 suppresses *N. benthamiana* resistance to *P. capsici*.

PvRXLR159-GFP and GFP were transiently expressed in *N. benthamiana*. Leaves were detached at 48 h, followed by inoculation with *P. capsici*. Images of typical symptoms were taken at 3 days after inoculated with *P. capsici* as shown in A. Quantification of lesion size in A was shown in B. Error bars represent the standard errors from three biological replicates. Asterisks represent significant differences from GFP (**P < .01, Student's t-test). Expression of PvRXLR159-GFP was checked by western blot as shown in C. PS, Ponceau S staining.

PvRXLR159 to shed light on the detail mechanism behind its interference effect on host resistance.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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