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A *Phytophthora capsici* virulence effector associates with NPR1 and suppresses plant immune responses

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Abstract

Salicylic acid (SA) plays a crucial regulatory role in plant immunity. NPR1 (non-expressor of pathogenesis related-1) is a SA receptor and plays a pivotal role in SA signaling. However, pathogen effectors which target NPR1 to promote infection have rarely been reported. Here, we identified a *Phytophthora capsici* effector RxLR48 that associates with NPR1, facilitates *P. capsici* infection and is required for pathogen virulence. Furthermore, we demonstrated that RxLR48 promotes NPR1's nuclear localization and inhibits its proteasome-mediated degradation, suggesting that RxLR48 suppresses SA signaling by targeting the central regulator NPR1. In addition, we showed that RxLR48 also suppresses pattern-triggered immunity (PTI). Together, our research indicates that *P. capsici* suppresses plant immunity by targeting SA and PTI pathways.

Keywords: *Phytophthora*, *Arabidopsis thaliana*, NPR1, Effector, Virulence, PTI

Background

The plant pathogenic oomycetes, which are evolutionarily close to algae in the kingdom Stramenopila, represent one of the greatest threats to agriculture and natural ecosystems in the worldwide (Kamoun 2003). Within oomycetes, *Phytophthora* that literally means plant destroyer, contains the most devastating pathogens causing notorious plant diseases (Tyler et al. 2006). For example, *Phytophthora infestans*, the causal agent of potato late blight, caused the Great Irish Famine in nineteenth century (Kroon et al. 2012). *P. sojae* causes soybean root and stem rot, leading to serious yield losses every year (Tyler 2007). *P. ramorum*, which affects trees and shrubs, is the most destructive disease of oaks worldwide (Kamoun et al. 2015). Thus, there are urgent needs to understand the infection process and mechanism of *Phytophthora* pathogens. *P. capsici* causes many devastating diseases on a broad range of plant species, including the model plant *Arabidopsis thaliana*, *Nicotiana benthamiana* and hundreds of vegetable crops such as pepper and cucumber (Lamour et al. 2012a; Wang et al. 2013). In recent years, the exploitation of *P. capsici* as a model pathogen is widely accepted and the molecular interaction between *P. capsici* and its hosts attracts increasing attentions (Wang et al. 2013).

Plant pathogens deliver molecules termed effectors to manipulate host immunity during infection, usually by targeting vital immune components or physiological processes. *Phytophthora* pathogens secrete hundreds of effectors, a prominent category among which is RxLR effectors. The RxLR effectors contain a conserved RxLR (Arg-any amino acid-Leu-Arg) motif in the N-terminal for translocation into plant cells (Tyler et al. 2006; Whisson et al. 2007; Dou et al. 2008a). Therefore, numerous researches have been implemented to uncover the biological function of effectors by identifying their host targets. For instance, *P. infestans* effector AVR3a can bind to and stabilize potato E3 ubiquitin ligase CMPG1 to prevent INF1-mediated cell death (Bos et al. 2010). Another *P. infestans* effector PexRD2 interacts with the kinase domain of potato MAPKKKε to perturb immunity-related signaling pathways (King et al. 2014). In addition, *P. sojae* effector PSR1 directly targets host PINP1, which is required for accumulation of distinct classes of endogenous small RNAs, to promote infection (Qiao et al. 2015). Like

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other *Phytophthora* species, *P. capsici* produces about four hundred cytoplasmic RxLR effectors (Lamour et al. 2012b). However, few studies have identified the host targets of these *P. capsici* RxLR effectors and the molecular mechanisms involved are still largely unknown.

To cope with pathogen infection, plants develop two layers of immune system to recognize pathogen signatures and activate defense responses at a molecular level (Jones and Dangl 2006). The first layer is dependent on host pattern recognition receptors (PRRs) through the recognition of conserved microbial features, leading to pattern-triggered immunity (PTI) that provides basal resistance to a wide range of pathogens (Jones and Dangl 2006). Plants also evolve intracellular receptors to specifically recognize corresponding avirulence effectors and activate effector-triggered immunity (ETI), resulting in robust disease resistance to certain pathogens (Cui et al. 2015).

SAR (systemic acquired resistance) is usually induced in distal tissues upon the onset of ETI at the primary infection sites, protects plants from secondary infection by activating long-lasting (up to several months) and broad-spectrum resistance (Kachroo and Robin 2013). Salicylic acid (SA) is a key plant hormone that is required for both local and systemic acquired resistance (Dong 2004). Plants that are defective in SA synthesis/accumulation always exhibit enhanced susceptibility to pathogens (Wildermuth et al. 2001). And the SA receptor NPR1 (non-expressor of pathogenesis related-1), which functions as a transcriptional co-activator, acts as the central signaling regulator during SAR (Mukhtar et al. 2009).

NPR1 was first cloned using a map-based approach in *Arabidopsis* and was found to encode a novel protein containing ankyrin repeats (Cao et al. 1997). Subsequent studies demonstrated that nuclear localization of NPR1 is essential for its activity in inducing *PR* genes and NPR1-mediated DNA binding of a basic leucine zipper transcription factor TGA2 is critical for activation of defense genes (Despres et al. 2000; Kinkema et al. 2000). Normally, NPR1 is present in the cytoplasm as an oligomer through intermolecular disulfide bonds. However, SAR results in reduction of NPR1 to a monomeric form, which accumulates in the nucleus to activate gene expression (Mou et al. 2003). S-nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) facilitates its oligomerization, while the SAR-induced NPR1 monomerization is catalyzed by thioredoxins (TRXs) (Tada et al. 2008). In addition, proteasome-mediated turnover of phosphorylated NPR1 through a Cullin3-based ubiquitin ligase in the nucleus is required for full induction of target genes and establishment of systemic immunity (Spoel et al. 2009). The systemic immunity also requires SnRK2.8-mediated phosphorylation of NPR1, which is necessary for the nuclear import of NPR1 (Lee et al. 2015). Recent study showed that sumoylation of NPR1 activates gene expression by switching its association with the WRKY transcription repressors to TGA transcription activators (Saleh et al. 2015). Considering NPR1 is a master regulator of SA-mediated responses, we proposed that *Phytophthora* pathogens would had evolved certain effectors to target host NPR1 for virulence function.

In this study, we screened *P. capsici* effectors by using NPR1 as a bait in yeast two-hybrid (Y2H) system and obtained an interacting effector RxLR48. We confirmed the interaction between RxLR48 and NPR1 by Y2H and co-immunoprecipitation (co-IP) assays. We found that transient expression of *RxLR48* facilitates *P. capsici* infection, and silencing of *RxLR48* impairs the pathogenicity of *P. capsici*. Furthermore, we demonstrated that co-expression of *NPR1* with *RxLR48* results in enhanced localization of NPR1 in the nucleus and elevated protein accumulation of NPR1 by inhibiting its proteasome-mediated degradation. Finally, we found that RxLR48 could suppress PTI-related immune responses and reduce the expression levels of PTI marker gene *FRK1*. Together, our results revealed that the *P. capsici* effector RxLR48 could target plant NPR1 and PTI signaling pathway for virulence and suggested potential mechanisms underlying this interaction to manipulate plant immunity.

Results

RxLR48 interacts with NPR1

To identify *P. capsici* effectors targeting plant NPR1, we exploited yeast two-hybrid approach to screen *P. capsici* RxLR effectors by using *Arabidopsis* NPR1 as a bait. Fourty-two RxLR effectors from *P. capsici* (Lamour et al. 2012b) were separately cloned into Y2H prey vector pGADT7 (Additional file 1: Table S1), and used for Y2H screening. Finally, we identified RxLR48 associating with plant NPR1.

To confirm this association, we cloned *RxLR48* into the bait vector pGBKT7, and *NPR1* into the prey vector pGADT7 for reciprocal Y2H assay. The result clearly showed that RxLR48 associates with NPR1 in yeast (Fig. 1a). To further confirm the association between RxLR48 and NPR1, we performed co-IP assay in *Arabidopsis* protoplasts. RxLR48-FLAG and NPR1-HA were transiently expressed in *Arabidopsis* protoplasts, and then the protoplasts were subsequently subjected to co-IP assay. A specific signal for NPR1-HA was clearly observed in the RxLR48-FLAG immunoprecipitate (Fig. 1b), indicating that RxLR48 associates with NPR1 *in planta*.

We also found that RxLR48 homologs were present in other tested *Phytophthora* species (Additional file 2: Figure S1). This result indicates that RxLR48 is a conserved effector in *Phytophthora* pathogens.

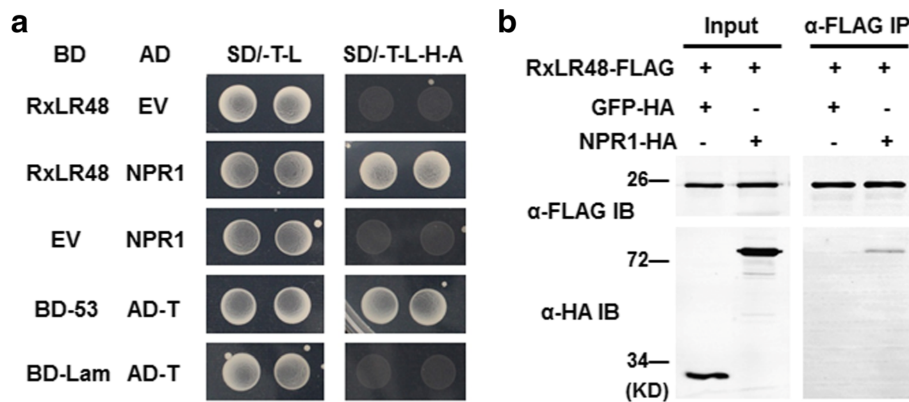

Fig. 1 RxLR48 interacts with NPR1. **a** Interaction between RxLR48 and NPR1 in yeast. *RxLR48* was cloned into bait plasmid pGBKT7 (BD) and *NPR1* was cloned into prey plasmid pGADT7 (AD), respectively. Combination of BD-53 and AD-T was used as a positive control while BD-Lam and AD-T as a negative control. Yeast transformants were grown on selective medium (SD) lacking Tryptophan (T) and Leucine (L) and selected on SD lacking Tryptophan (T), Leucine (L), Histidine (H) and Adenine (A). The plates were photographed at 3 days after inoculation. **b** Interaction between RxLR48 and NPR1 in *Arabidopsis*. *RxLR48-FLAG* and *NPR1-HA* were transiently co-expressed in *Arabidopsis* protoplasts. RxLR48 together with GFP was used as a negative control. Total proteins were extracted, followed by immunoprecipitation with an α-FLAG antibody (α-FLAG IP). Input and the bound proteins were detected via immunoblot using α-FLAG and α-HA antibodies, respectively

RxLR48 enhances *P. capsici* infection in *N. benthamiana*

To investigate RxLR48's virulence function, *GFP-RxLR48* was transiently expressed in *N. benthamiana* and GFP alone was used as a negative control. The infiltrated leaves were then inoculated with *P. capsici*. Expression of *RxLR48* significantly promoted infection of *P. capsici* (Fig. 2a, b). The photograph of disease symptoms and statistical analysis of lesion diameters together showed that ectopic expression of *RxLR48* led to development of bigger lesions, compared with the *GFP* expression control (Fig. 2a, b). The GFP-RxLR48 fluorescence was observed in the infiltrated leaves using a confocal microscope to ensure protein expression (Fig. 2c). Together, these results demonstrated that RxLR48 is able to effectively enhance *P. capsici* colonization *in planta.*

RxLR48 contributes to pathogen virulence

To explore whether RxLR48 contributes to pathogen virulence, we silenced *RxLR48* in *P. capsici*. To check silencing efficiency, total RNA was extracted from independent transformants and the transcription levels of *RxLR48* were measured by quantitative real-time PCR (qRT-PCR). We obtained two *RxLR48*- silenced transformants, named T12 and T129, whose transcription levels were significantly decreased to 0.5% and 3.8% of the wild-type strain LT263, respectively (Fig. 3a). An additional transformant, T108, was selected as a control in which *RxLR48* expression remained unaffected (Fig. 3a). Next, we evaluated the virulence of the *RxLR48*-silenced transformants on *N. benthamiana* leaves. The detached *N. benthamiana* leaves were drop-inoculated with zoospore suspensions of T12 or T129 on the right side while

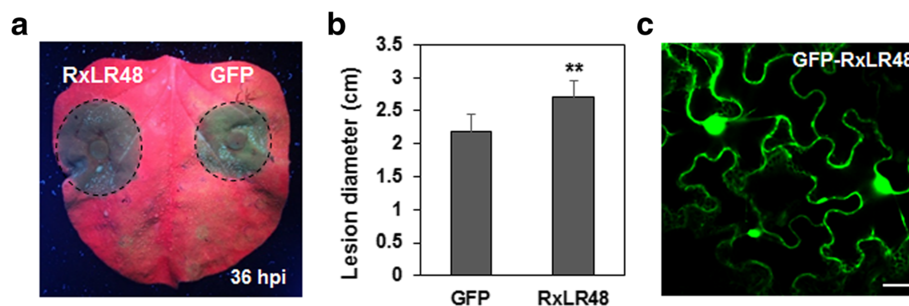

Fig. 2 RxLR48 facilitates *Phytophthora capsici* infection in *Nicotiana benthamiana*. **a, b** Enhanced infection of *P. capsici* by RxLR48. Equal area of mycelium was inoculated onto leaf regions transiently expressing *RxLR48* or *GFP* at 36 hpi. Photographs (**a**) were taken under UV light and lesion diameters were measured at 36 hpi. The shown data (**b**) calculated from three independent biological replicates, and error bars represent + SD of at least six leaves each (**, $P < 0.01$, Student's t test). **c** Expression of *RxLR48 in planta*. Expression of *RxLR48* was visualized by confocal images of *N. benthamiana* epidermal cells. Photographs were taken at 48 hpi. White scale bar represents 20 μm

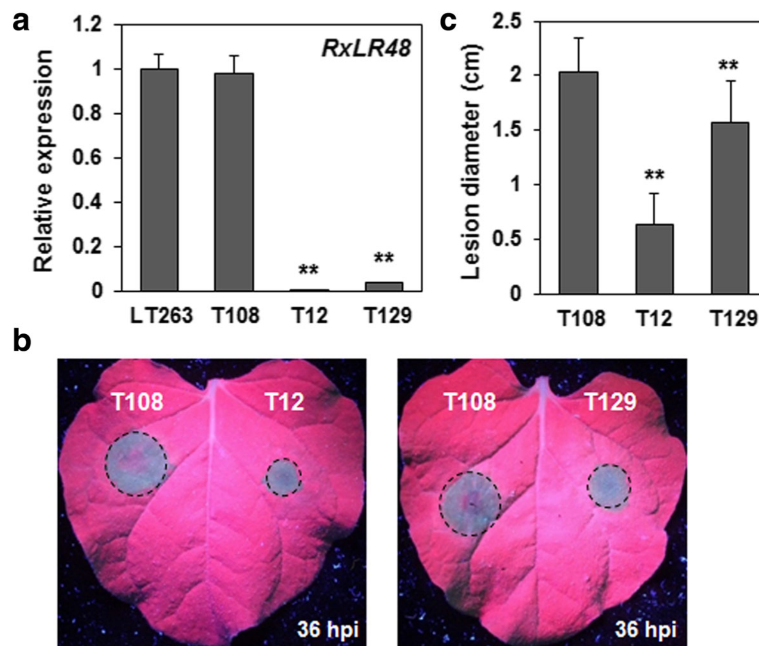

Fig. 3 RxLR48 contributes to *Phytophthora capsici* virulence. **a** Relative expression levels of *RxLR48* in *P. capsici* transformants. Total RNA was isolated and transcript levels of *RxLR48* were determined by qRT–PCR with *P. capsici tubulin* gene as a pathogen reference. T108 was a non-silenced transformant carrying the same silencing construct. (**, $P < 0.01$ compared with LT263, Dunnett's test). **b**, **c** Inoculation of *RxLR48*-silenced transformants. Leaves of *Nicotiana benthamiana* were inoculated with the non-silenced transformant T108 (control) and *RxLR48*-silenced transformants T12 and T129. Photographs (**b**) were taken under UV light and lesion diameters were measured at 36 hpi. The shown data (**c**) are expressed as the mean + SD of three replicates. Asterisks indicate significant differences (**, $P < 0.01$ compared with T108, Dunnett's test)

T108 zoospores on the left side (Fig. 3b). T12 and T129 developed smaller lesion diameters compared to T108 36 h post inoculation (hpi) (Fig. 3b). Meanwhile, statistical analysis showed that the lesion diameters caused by T12 and T129 were reduced to 31% and 77% relative to that caused by T108, respectively (Fig. 3c). Thus, these results indicate that RxLR48 is required for pathogen infection.

RxLR48 promotes nuclear accumulation of NPR1

It was reported that NPR1 was primarily localized in the cytoplasm and nucleus under normal circumstances, while accumulated in the nucleus in response to SA signal (Kinkema et al. 2000). To investigate whether RxLR48 could alter the subcellular localization of NPR1, GFP-NPR1 and mCherry-RxLR48 were transiently expressed in *N. benthamiana*. GFP-NPR1 distributed in the cytoplasm and nucleus when expressed alone *in planta* (Fig. 4a), and mCherry-RxLR48 exhibited similar subcellular localization as NPR1 (Fig. 4b). When mCherry was co-expressed, GFP-NPR1 showed similar localization pattern as GFP-NPR1 alone (Fig. 4c). Interestingly, the fluorescence intensity of NPR1 was significantly enhanced in the nucleus when mCherry-RxLR48 was co-expressed (Fig. 4d). Hence, our results suggest that RxLR48 could alter the subcellular localization of NPR1 by promoting its nuclear accumulation.

RxLR48 inhibits protein degradation of NPR1

Previous studies reported that proteasome-mediated turnover of NPR1 plays an important role in modulating transcription of its target genes (Spoel et al. 2009). In order to test whether RxLR48 could affect the stability of NPR1 during interaction, *RxLR48-FLAG* and *NPR1-HA* were transiently co-expressed in *Arabidopsis* protoplasts, and protein accumulation of NPR1-HA was detected by immunoblot. Results showed that the abundance of NPR1 was significantly higher when co-expressed with *RxLR48* compared to *NPR1* alone (Fig. 5a). It was shown that SA treatment induces NPR1 monomer formation in the nucleus, resulting in an actual increase in total NPR1 protein (Spoel et al. 2009). We treated *NPR1*-expressing protoplasts with SA, and immunoblot results proved that the abundance of NPR1 was greatly elevated regardless of the presence of RxLR48 (Fig. 5a). Together, these results demonstrated that co-expression of RxLR48 increases the accumulation of NPR1 protein.

Next, we analyzed NPR1 protein accumulation in the presence of MG132, an inhibitor of 26S proteasome

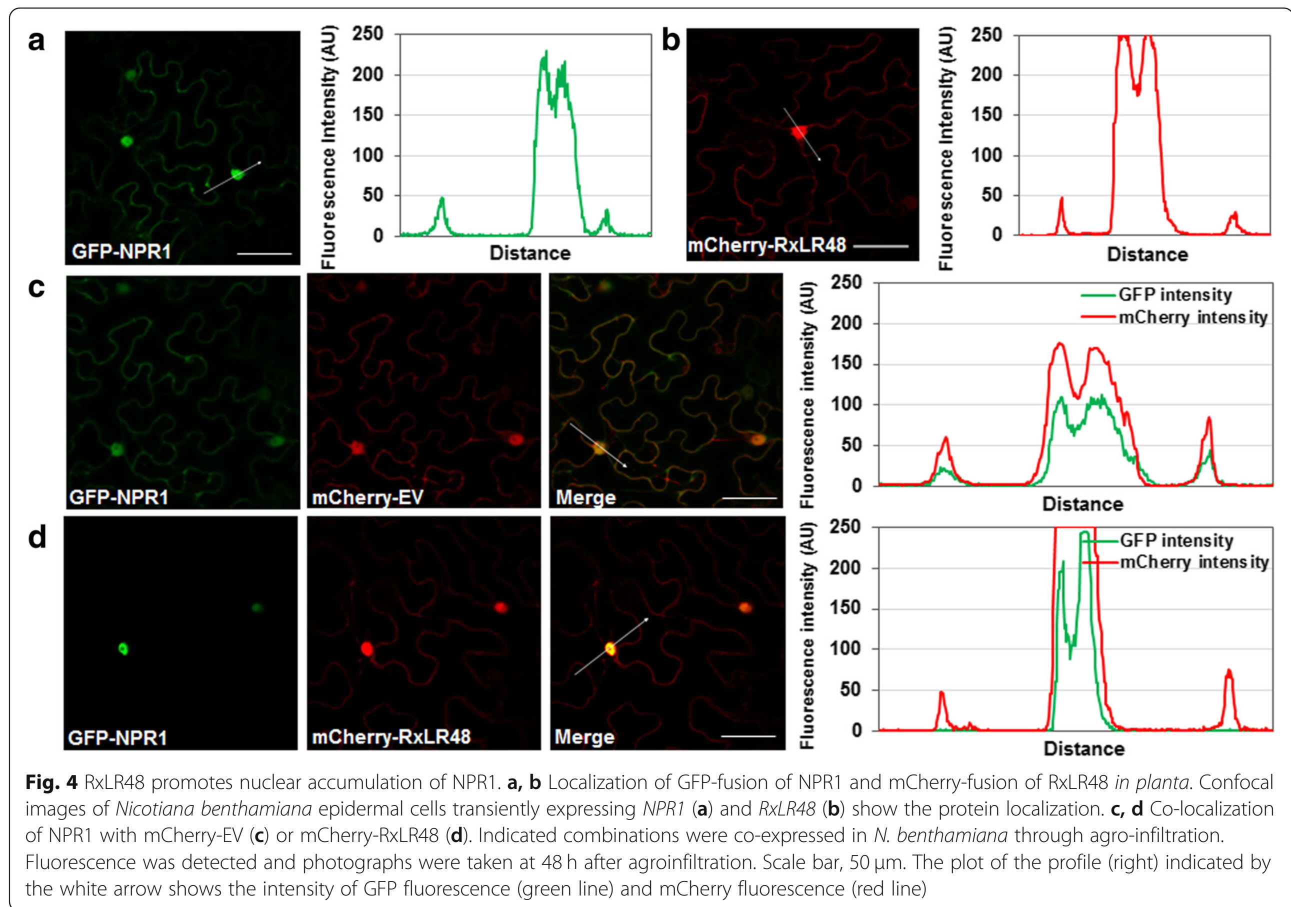

Fig. 4 RxLR48 promotes nuclear accumulation of NPR1. **a, b** Localization of GFP-fusion of NPR1 and mCherry-fusion of RxLR48 *in planta*. Confocal images of *Nicotiana benthamiana* epidermal cells transiently expressing *NPR1* (**a**) and *RxLR48* (**b**) show the protein localization. **c, d** Co-localization of NPR1 with mCherry-EV (**c**) or mCherry-RxLR48 (**d**). Indicated combinations were co-expressed in *N. benthamiana* through agro-infiltration. Fluorescence was detected and photographs were taken at 48 h after agroinfiltration. Scale bar, 50 μm. The plot of the profile (right) indicated by the white arrow shows the intensity of GFP fluorescence (green line) and mCherry fluorescence (red line)

(Zhang et al. 2015). When expressing NPR1 alone, the conspicuously elevated protein accumulation of NPR1 was observed after MG132 treatment (Fig. 5b), consistent with previous findings that NPR1 was degraded through the 26S proteasome (Spoel et al. 2009). Moreover, immunoblot analysis revealed that the abundance of NPR1 remained the same upon MG132 treatment regardless of the presence of RxLR48, whereas the negative control of dimethyl sulfoxide (DMSO) treatment showed similar result to Fig. 5a. Taken together, these results

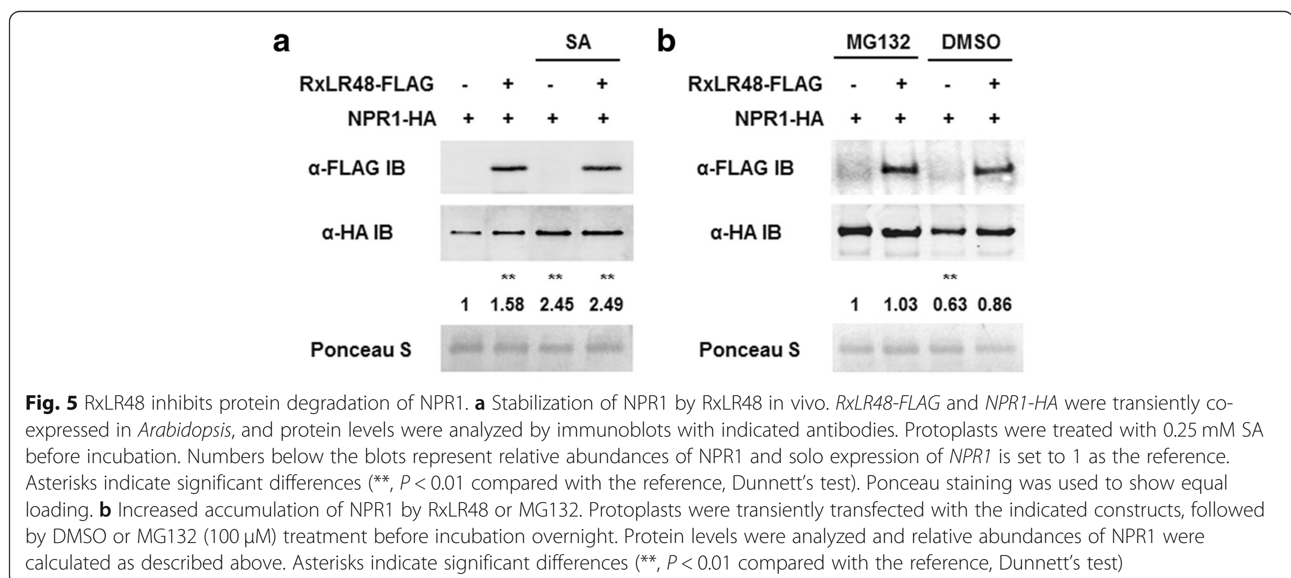

Fig. 5 RxLR48 inhibits protein degradation of NPR1. **a** Stabilization of NPR1 by RxLR48 in vivo. *RxLR48-FLAG* and *NPR1-HA* were transiently co-expressed in *Arabidopsis*, and protein levels were analyzed by immunoblots with indicated antibodies. Protoplasts were treated with 0.25 mM SA before incubation. Numbers below the blots represent relative abundances of NPR1 and solo expression of *NPR1* is set to 1 as the reference. Asterisks indicate significant differences (**, $P < 0.01$ compared with the reference, Dunnett's test). Ponceau staining was used to show equal loading. **b** Increased accumulation of NPR1 by RxLR48 or MG132. Protoplasts were transiently transfected with the indicated constructs, followed by DMSO or MG132 (100 μM) treatment before incubation overnight. Protein levels were analyzed and relative abundances of NPR1 were calculated as described above. Asterisks indicate significant differences (**, $P < 0.01$ compared with the reference, Dunnett's test)

indicate that RxLR48 is able to promote NPR1 protein accumulation by suppressing its proteasome-mediated degradation.

RxLR48 suppresses ROS production and callose deposition in plants

In addition to interfering with SA signaling by affecting NPR1 subcellular localization and protein accumulation, we want to clarify if RxLR48 also suppresses PTI responses. Reactive oxygen species (ROS) production is a typical response in PTI signaling pathway (Dodds and Rathjen 2010). To test if RxLR48 is able to suppress ROS production, we conducted inoculation assay using the *RxLR48*-silenced transformant. The ROS production was visualized by DAB staining. Compared with the T108 control, inoculation of the silencing transformant T12 resulted in obviously elevated H_2O_2 accumulation in *N. benthamiana* leaves at 9 hpi (Fig. 6a). Callose deposition is another typical response in PTI signaling and is regulated by ROS (Dodds and Rathjen 2010). The leaves challenged with the silencing transformant T12 showed significantly enhanced callose deposition compared with T108 control (Fig. 6b). Taken together, these results suggest that RxLR48 suppresses PTI-related immune responses.

RxLR48 down-regulates the expression of PTI-responsive gene *FRK1*

To further clarify the virulence function of RxLR48, we subsequently detected the expression of defense-related genes in *RxLR48*-expressing *Arabidopsis* protoplasts. First, *RxLR48* and EV (empty vector) were transiently expressed in protoplasts, then total RNA was isolated from protoplasts for qRT-PCR analysis. Four defense-related genes were selected for qRT-PCR analysis (Fig. 7a). NPR3, a paralog of NPR1, acts as a receptor for the immune signal SA (Fu et al. 2012); *PR1* (pathogenesis-related 1), is a marker gene in SA signaling pathway (Cao et al. 1997); *FRK1* has been widely used as a PTI marker gene (Li et al. 2005). The expression of *NPR1 and NPR3* showed no significant differences in the *RxLR48*-expressing protoplasts compared to that in the EV control (Fig. 7a). We constantly observed reduced *PR1* expression in the *RxLR48*-expressing protoplasts compared with the EV control, although it is not statistically significant when we set $P < 0.01$. Interestingly, transient expression of *RxLR48* resulted in the reduction of *FRK1* transcripts to 37% relative to that in the EV negative control (Fig. 7a). This result further confirmed that RxLR48 is able to suppress PTI signaling.

MAPK activation is considered as an early biochemical event of PTI signaling pathways (Zhang et al. 2010; Segonzac and Zipfel 2011; Feng et al. 2012). To test if RxLR48 also affects MAPK activation in host cells, protoplasts transiently expressing EV, *RXLR48* and *HopAI1* were treated with a 22-amino acid peptide conserved in the N-terminus of bacterial flagellin (flg22) at the indicated time points. The expression of *RxLR48* was unable to prevent phosphorylation of MAPKs upon flg22 treatment, compared with that in the EV control (Fig. 7b). In contrast, *Pseudomonas syringae* effector HopAI1, the positive control, completely blocked MAPKs activation as reported (Zhang et al. 2007) (Fig. 7b). These results suggest that RxLR48 mainly suppresses ROS production but not MAPK cascades.

Discussion

NPR1 plays an important role in SAR and plant immunity. However, how pathogen effectors target NPR1 to diminish SA-dependent signaling pathway remained a mystery until recently. An impressive recent research reported that the *P. syringae* type III effector AvrPtoB, a U-box type E3 ubiquitin ligase, mediates the degradation of NPR1 via host 26S proteasome, resulting in disrupting SA signaling, pattern-triggered immunity and systemic acquired resistance (Chen et al. 2017). In addition, a

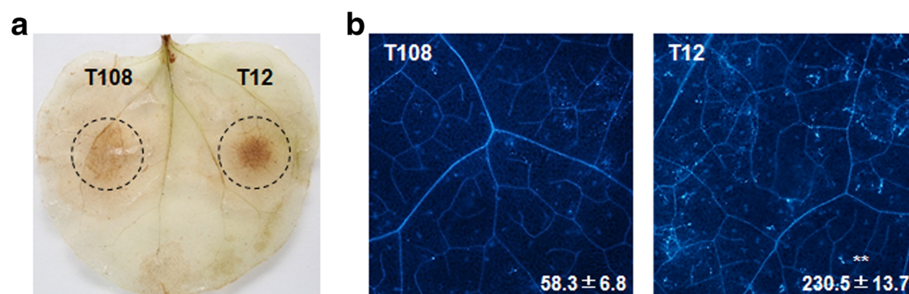

Fig. 6 RxLR48 contributes to suppression of PTI-related immune responses. **a** H_2O_2 accumulation during *Phytophthora capsici* infection. *RxLR48*-non-silenced transformant T108 (control) and silenced transformant T12 were inoculated on *Nicotiana benthamiana* detached leaves. DAB staining was performed at 9 hpi. The black dashed circle indicates inoculation sites. **b** Callose deposition after *P. capsici* infection. Detached leaves of *N. benthamiana* were inoculated as described above and then stained by aniline blue at 12 hpi. Values of callose deposition shown were quantitated by using ImageJ software (mean ± SD; $n \geq 6$; **, $P < 0.01$, Student's t test)

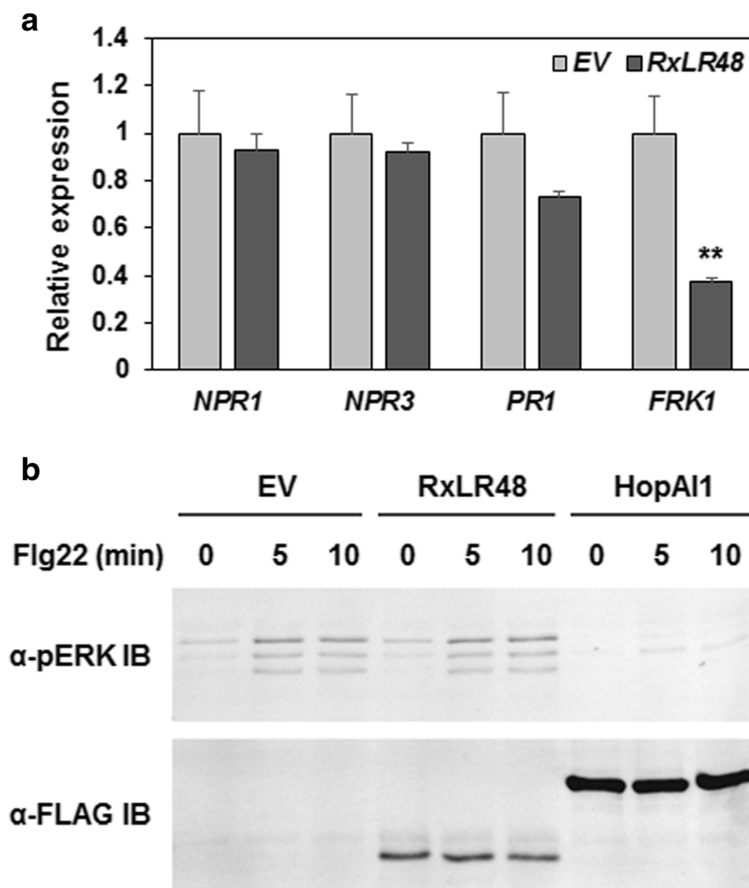

Fig. 7 RxLR48 down-regulates expression of *FRK1*. **a** Transcription levels of *NPR1*-related genes and *FRK1*. Protoplasts were transiently transfected with the indicated constructs. For detection of *NPR1*, *NPR3* and *PR1* gene expression, protoplasts were harvested directly after incubation; For *FRK1* detection, protoplasts were treated with 1 μM flg22 for 8 h before harvest. Real-time RT-PCR were carried out to analyze relative expression of the genes in *Arabidopsis* with the *actin* gene as a reference. Each data represents the mean of three replicates. Error bars indicate standard deviation. Asterisks indicate significant differences (**, $P < 0.01$, Student's t test). **b** Flg22-induced MAPK activation. Protoplasts were transfected with empty vector, RxLR48 and HopAI1 (positive control), and treated with 1 μM flg22 at the indicated time points before protein extraction. Total proteins were analyzed by immunoblot with Phospho-p44/42 MAPK antibody (α-pERK)

conserved rust protein from *Puccinia striiformis* interacts with wheat NPR1 in the nucleus, and competes with NPR1's interaction partner TGA2.2 in barley (Wang et al. 2016). In this study, we adopted the Y2H system to screen the candidate effectors of *P. capsici* by using NPR1 as a bait. We identified an NPR1-interacting effector RxLR48 and confirmed the interaction by Y2H and co-IP assays. Considering subcellular localization and protein stability of NPR1 are important for its function, we successively demonstrated that RxLR48 affects these aspects to interfere with SA signaling pathway.

Previous studies reported that some pathogen effectors can suppress plant immunity, termed as effector triggered susceptibility (ETS), thereby enhancing pathogenicity by using diversified strategies in susceptible hosts (Gohre and Robatzek 2008). One of the familiar strategies is to alter subcellular localization of their host targets. For example, the *P. infestans* RxLR effector PITG_03192 interacts with two putative NAC transcription factors StNTP1 and StNTP2 at the endoplasmic reticulum (ER) membrane, and prevents culture filtrate-triggered re-localization of StNTP1 and StNTP2 from entering nucleus (McLellan et al. 2013). Furthermore, a potential effector SsSSVP1 from *Sclerotinia sclerotiorum* interacts with QCR8, and disturbs its subcellular localization by hijacking QCR8 to the cytoplasm before targeting to mitochondria (Lyu et al. 2016). In this study, we found that co-expression of *NPR1* with *RxLR48* resulted in enhanced localization of NPR1 in the nucleus, which would break the dynamic balance of the distribution ratio of NPR1. Considering NPR1 is present in the cytoplasm as an oligomer in non-active state, while monomerized and translocted into the nucleus to activate the expression of *PR* genes in the presence of pathogen or SA treatment (Mou et al. 2003). We hypothesized that RxLR48 interfered with distribution of NPR1, leading to aberrant SA-mediated defense response, which needs to be further elucidated in the future.

Accumulating evidence from prior studies supports that post-translational regulation of target proteins, including interfering with the stability of target proteins, is a commonly used strategy utilized by a large variety of pathogens. For example, a type III secretion system effector HopX1 from *P. syringae* pv. *tabaci* interacts with and promotes the degradation of JAZ (JASMONATE ZIM DOMAIN) proteins, a key family of jasmonate (JA)-repressors, to enhance the activation of JA-mediated signaling and susceptibility in *Arabidopsis* (Gimenez-Ibanez et al. 2014). Another effector Tin2 secreted by *Ustilago maydis* promotes virulence by refueling anthocyanin biosynthesis in maize. Tin2 stabilizes a maize protein kinase ZmTTK1, leading to rewiring metabolites into the anthocyanin pathway to lower their availability for other defense responses (Tanaka et al. 2014). In our study, ectopic expression of *RxLR48* resulted in elevated protein accumulation of NPR1 by inhibiting 26S proteasome-mediated degradation. It was reported that proteasome-mediated turnover of NPR1 in the nuclear is required for full induction of target genes and establishment of systemic immunity (Spoel et al. 2009). Thus, our results suggested that RxLR48 blocks proteasome-mediated degradation of NPR1, which is in accord with an excess of nuclear NPR1.

To further elucidate the role of RxLR48 during infection, we also checked the transcription levels of the *NPR1*-related genes and *FRK1*. Although RxLR48 promotes total and nuclear accumulation of NPR1, relative expression of *PR1* was not significantly affected. Instead, the transcription level of *FRK1* was subverted by transient expression of *RxLR48*. Considering our findings that RxLR48 contributes to the suppression of PTI responses including H_2O_2 production and callose deposition, we assumed that RxLR48 is involved in obstructing PTI signaling pathway. A number of pathogen effectors suppress flg22-triggered immunity by altering PTI signaling pathway at different stages. For example, the *P. infestans* RxLR effector AVR3a associates with the Dynamin-Related Protein 2 (DRP2) to suppress the endocytosis of the activated FLS2 receptor (Chaparro-Garcia et al. 2015). *Xanthomonas campestris* pv *campestris* type III effector AvrAC specifically uridylylates BIK1 (BOTRYTIS-INDUCED KINASE1) and RIPK (RPM1-INDUCED PROTEIN KINASE) to reduce phosphorylation of these two receptor-like cytoplasmic kinases (Feng et al. 2012). In our study, flg22-induced MAPK activation remained unaffected when *RxLR48* was transiently expressed in *Arabidopsis* protoplasts, suggesting that RxLR48 acts independent or downstream of the MAPK cascades to suppress PTI signaling. This is reminiscent of another *Phytophthora* effector PsCRN63 (Li et al. 2016). Therefore, additional experiments are required to clarify which stages RxLR48 interferes with PTI.

Conclusions

In summary, we identified a virulence effector RxLR48 from the hemibiotrophic oomycete pathogen *P. capsici*. RxLR48 not only interferes with plant immunity by associating with and altering NPR1's protein localization and accumulation, but also suppresses PTI responses by inhibiting PTI-responsive genes. This study provides a new insight into the mechanisms of oomycete pathogen virulence.

Methods

DNA constructs

For Y2H assay, *RxLR48* (lacking the signal peptide-encoding region) was PCR-amplified from *P. capsici* strain LT263 genomic DNA and *NPR1* coding region was amplified by using *A. thaliana* complementary DNA as templates. Then the corresponding PCR products were inserted into pGBKT7 or pGADT7, respectively. To generate constructs for protoplast transfection, coding sequences of indicated genes were amplified and inserted into the pUC19-35S-FLAG/HA-RBS vector (Li et al. 2005). For transient expression in *N. benthamiana*, *RxLR48* was PCR-amplified and cloned into pBinGFP2 or pBinPLUS::mCherry vector under the control of the 35S promoter (Song et al. 2015). And *NPR1* was amplified and cloned into pBinGFP2 as well. For transformation of *P. capsici*, *RxLR48* was amplified and cloned into pHam34 which was maintained in our laboratory. All primers used for PCR amplification are listed in Additional file 1: Table S2.

Yeast two-hybrid assay

The yeast (*Saccharomyces cerevisiae*) two-hybrid assay was performed according to the Yeast Protocols Handbook (Clontech) using the Y2H Gold yeast reporter strain (Clontech). Yeast cells were co-transformed with the indicated plasmid combinations, and the transformed yeast cells were selected using synthetic dropout (SD/−Leu/−Trp) medium, then further transferred to SD/−Leu/−Trp/-His/−Ade selective medium for growth analysis.

Plant materials and growth

The Columbia ecotype of *A. thaliana* was used for protoplast isolation. The plants were grown at 23 °C with a 10-h-light/14-h-dark photoperiod for 4 to 5 weeks. For transient expression, *N. benthamiana* plants were grown and maintained in a greenhouse for 4–6 weeks at 25 °C or 20 °C under a 16 h/8 h day/night photoperiod.

Arabidopsis protoplast transfection and protein extraction

Protoplast isolation and transfection were implemented as described (Li et al. 2005), except that the transfected protoplasts were incubated in W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES pH

5.7) instead of 0.4 M mannitol. Total protein was isolated with an extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM EDTA, 0.3% Triton-X 100, 1 mM DTT, complete protease inhibitors (Roche).

Co-IP assay

Arabidopsis protoplasts were transfected with indicated plasmid combinations and incubated overnight, followed by protein extraction. The α-FLAG IP was carried out as previously described (Li et al. 2016). Total protein and immune precipitates were separated by SDS–PAGE (SDS–polyacrylamide gel electrophoresis), and detected by immunoblots with α-FLAG antibody (Sigma-Aldrich) together with α-HA antibody (Tiangen), respectively.

Transient expression in *N. benthamiana*

The *A. tumefaciens* strain GV3101 in our lab was used for this experiment (Liu et al. 2011). For infiltration, recombinant strains were cultured overnight at 28 °C in Luria-Bertani medium with the antibiotics kanamycin and rifampicin. The cells were harvested by centrifugation at 5600 x g and washed three times in 10 mM $MgCl_2$, and then resuspended in *Agro*-infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone) to an appropriate optical density (OD) at 600 nm (0.2). For co-expression, suspensions carrying each construct were thoroughly mixed before infiltration.

P. capsici culture conditions and inoculation assay

The *P. capsici* strain LT263 used in the study were routinely cultured at 25 °C in the dark on 10% (*v*/*v*) V8 juice medium. For inoculation on *N. benthamiana*, mycelial plugs were inoculated on one-half of the leaves at 36 h after infiltration. Inoculated leaves were photographed under UV light and lesion diameters were measured at 36 hpi. This assay was repeated at least three times.

Zoospores were obtained by incubating mycelial plugs in 10% (v/v) V8 broth at 25 °C for 3 days, then washed three times with sterilized water at room temperature, numerous sporangia were developed after incubation overnight in sterilized water. To initiate zoospore release, the cultures were transferred into fresh cold water (4 °C) for 0.5 h followed by incubation at 25 °C for 1 h. The concentrations of zoospores were estimated with a hemacytometer. Inoculation assay was performed using droplets of zoospore suspension (10 μL of a 50,000 zoospores/mL solution) and incubated in a growth room at 25 °C in darkness. Inoculated leaves were photographed under UV light and lesion diameters were measured at the indicated time points.

Confocal laser scanning microscopy

N. benthamiana leaf tissues expressing the corresponding constructs were mounted in water under a coverslip at 48 h after infiltration. Images were captured and processed using the Zeiss LSM 710 confocal laser scanning microscope. The GFP fluorescence were excited at 488 nm, and the mCherry fluorescence were excited at 561 nm.

P. capsici transformation and characterization

For transformation of *P. capsici*, we used the polyethylene glycol (PEG)-mediated protoplast transformation procedure as described (Dou et al. 2008b). Transformants appeared within 4–10 days in pea broth medium containing 30 μg/mL G418 (Sigma) at 25 °C under the dark conditions were multiplied further on 10% V8 solid medium containing 30 μg/mL G418 for selection of putative transformants. To check silencing efficiency, the transcript level of *RxLR48* gene of different transformants was measured by qRT-PCR.

RNA isolation and qRT-PCR

For detection of *NPR1*, *NPR3* and *PR1* gene expression, protoplasts were harvested directly after incubation; For *FRK1* detection, protoplasts were treated with 1 μM flg22 for 8 h before harvest. Total RNA was extracted from *P. capsici* mycelia or *Arabidopsis* protoplasts by using RNA-simple Total RNA Kit (Tiangen) according to the manufacturer's instructions. cDNA was synthesized by using the SuperScriptIII First-Strand Kit (Invitrogen). Real-Time PCR was performed on ABI Prism 7500 Fast Real-Time PCR system by using SYBR Premix Ex Taq Kit (TaKaRa) following manufacturer's instructions. The gene-specific primers used for qRT-PCR are listed in Additional file 1: Table S2.

DAB staining and callose deposition assay

For determination of H_2O_2 accumulation, *N. benthamiana* leaves were stained with 1 mg/mL DAB solution for 8 h in the dark at 10 hpi, and then destained with ethanol before observation by light microscopy. For measurement of callose deposition, *N. benthamiana* leaves were stained with aniline blue, and visualized with a fluorescence microscope as described (Song et al. 2015). The number of callose deposition was counted using Image J software and significant differences were identified by Student's t test.

MAPKs activity assay

Protoplasts were transfected with empty vector, RxLR48-FLAG and HopAI1-FLAG, and treated with 1 μM flg22 for 0, 5, 10 min before protein extraction. The protein concentration was determined using a Bio-Rad Bradford protein assay kit, and equal amounts of total protein were electrophoresed on 10% SDS-PAGE. An α-pERK antibody (no. 4370S, Cell Signaling) was used to determine the phosphorylation state of MPK3, MPK4 and MPK6.

Additional files

Additional file 1: Table S1. Screening of NPR1-interacted effectors in *Phytophthora capsici*. **Table S2.** Primers used in this study. (XLSX 28 kb)

Additional file 2: Figure S1. Phylogenetic relationships of RxLR48 and homologs in four *Phytophthora* species. The phylogenetic tree was constructed using MEGA 5 with the neighbor-joining method and 1000 bootstrap replicates. Ps, Pr, Pi, and Pc, correspond to *P. sojae*, *P. ramorum*, *P. infestans*, and *P. capsici*, respectively. (TIF 568 kb)

Abbreviations

BIK1: BOTRYTIS-INDUCED KINASE1; co-IP: Co-immunoprecipitation; DMSO: Dimethyl sulfoxide; DRP2: Dynamin-Related Protein 2; ER: Endoplasmic reticulum; ETI: Effector triggered immunity; ETS: Effector triggered susceptibility; *FRK1*: *FLG22-INDUCED RECEPTOR-LIKE KINASE 1*; GSNO: S-nitrosoglutathione; JA: Jasmonate; JAZ: JASMONATE ZIM DOMAIN; MAPK: Mitogen-activated protein kinases; NPR1: Non-expressor of pathogenesis related-1; *PR*: *Pathogenesis-related*; PRRs: Pattern recognition receptors; PTI: Pattern-triggered immunity; qRT-PCR: Quantitative real-time PCR; RIPK: RPM1-INDUCED PROTEIN KINASE; ROS: Reactive oxygen species; RxLR: Arg-any amino acid-Leu-Arg; SA: Salicylic acid; SAR: Systemic acquired resistance; TRXs: Thioredoxins; Y2H: Yeast Two-Hybrid

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Authors' contributions

QL, DD and MZ conceived and designed the research. QL, YC, JW, FZ, YJ and QZ performed the experiments. QL, DS and MZ analyzed the data. QL, YC, MJ and MZ wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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