Received: 3 September 2019 | Revised: 26 February 2020 | Accepted: 12 March 2020

DOI: 10.1111/mpp.12937

SHORT COMMUNICATION

Molecular Plant Pathology | WILEY

The *Ralstonia solanacearum* effector RipI induces a defence reaction by interacting with the bHLH93 transcription factor in *Nicotiana benthamiana*

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 31671988, 31701752, 31801696 and 31872919; Science Foundation of Fujian Provincial Science & Technology Department, Grant/Award Number: 2017J01434; Scientific Project of Fujian Provincial Education Department, Grant/Award Number: JA15152

Abstract

Ralstonia solanacearum releases a set of effectors into plant cells that modify the host defence reaction. The role of the effector protein RipI during infection has not been elucidated. In this study, we demonstrated that transient overexpression of RipI induces the hypersensitive response (HR), up-regulating the HR marker gene *hin1*, in *Nicotiana benthamiana*. Deletion of *R. solanacearum ripI* led to increased virulence in tomato (*Solanum lycopersicum*) plants. Through yeast two-hybrid and pull-down assays, we identified an interaction between the *N. benthamiana* transcription factor bHLH93 and RipI, both of which could be localized in the nucleus of *Arabidopsis* protoplasts. Silencing of *bHLH93* markedly attenuated the RipI-induced HR and induced expression of the *PDF1.2* defence gene. These data demonstrate that the *R. solanacearum* effector RipI induces a host defence reaction by interacting with the bHLH93 transcription factor.

KEYWORDS

bHLH93, defence reaction, *Ralstonia solanacearum*, RipI, transcription factor

Ralstonia solanacearum causes bacterial wilt in a wide range of host plants, including *Nicotiana benthamiana*. This pathogen is a species complex, a heterogeneous group of related but genetically distinct strains (Genin and Denny, 2012). The available genome sequences demonstrate that *R. solanacearum* possesses over 110 effector candidates that vary among isolates (Lonjon *et al.*, 2018), secreted by the type III secretion system (T3SS).

The first complete genomic sequence of *R. solanacearum* was characterized from the strain GMI1000 isolated from tomato (*Solanum lycopersicum*) (Salanoubat *et al.*, 2002). GMI1000 is pathogenic on the model plant *Arabidopsis thaliana* and nonpathogenic on *N. benthamiana*. The GMI1000 genome encodes around 72 effectors, one-third of which are conserved in all *R. solanacearum* strains (Peeters *et al.*, 2013). The RipP2 effector interacts with the *Arabidopsis* NB-LRR protein RRS1-R, leading to avirulence on *A. thaliana* Nd-1 (Deslandes *et al.*, 2003). RipP1 and RipAA act jointly to specify an incompatible interaction within *Nicotiana* plants (Poueymiro *et al.*, 2009). RipAY and RipAK target plant redox regulators and host catalases, respectively, suppressing the immune response (Sun *et al.*, 2017; Sang *et al.*, 2018). Furthermore, a few effectors induce hypersensitive response

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(HR)-like responses in different plant species. For example, RipAX2 induces an HR in eggplant (*Solanum torvum*) via an essential zinc-protease motif (Nahar *et al.*, 2014), and elicits the resistance of eggplant AG91-25 (Morel *et al.*, 2018).

A 47-kDa effector protein, RipI, composed of 430 amino acids is encoded by *RSc0041* in *R. solanacearum* GMI1000 (GenBank no. NC_003295.1) and contains two predicted motifs: an integrase motif from N54 to N174 and a DNA polymerase III subunit γ motif from N143 to N378 at the N-terminus of RipI, respectively (Figure S1). Ectopic expression of RipI in *Saccharomyces cerevisiae* results in cell apoptosis; the integrase motif is essential for the apoptosis (Deng *et al.*, 2016). To examine plant responses to RipI, we transiently expressed RipI in *N. benthamiana* leaves using *Agrobacterium*-mediated transformation. An HR was evident 2 days post-agroinfiltration, coupled with cell death and hydrogen peroxide accumulation (Figure 1a,b). Furthermore, the HR marker gene *hin1* (Gopalan *et al.*, 1996) was induced 17-fold relative to control leaves agroinfiltrated with empty binary vector (Figure 1c).

To investigate the role of the *R. solanacearum* RipI effector in virulence, we constructed the deletion mutant Δ*ripI* in strain GMI1000. Although wilt symptoms initially appeared at 3 days post-inoculation (dpi), tomato plants inoculated with Δ*ripI* displayed more rapid disease development than those inoculated with wild-type GMI1000. The disease index of Δ*ripI* was 20.1, while that of the wild type was only 8.3 at 3 dpi. By 6 dpi, all Δ*ripI*-infected plants had fully wilted, and the disease index of GMI1000 was 82.7 (Figure 1d). We observed wilt symptoms in the tomato plants inoculated with the Δ*ripI* strain complemented with *ripI* (Δ*ripI*:pBBR-RipI) at 4 dpi, 1 day later than observed in the wild type and mutant Δ*ripI*; however, the disease index of the complemented strain was lower than that of the

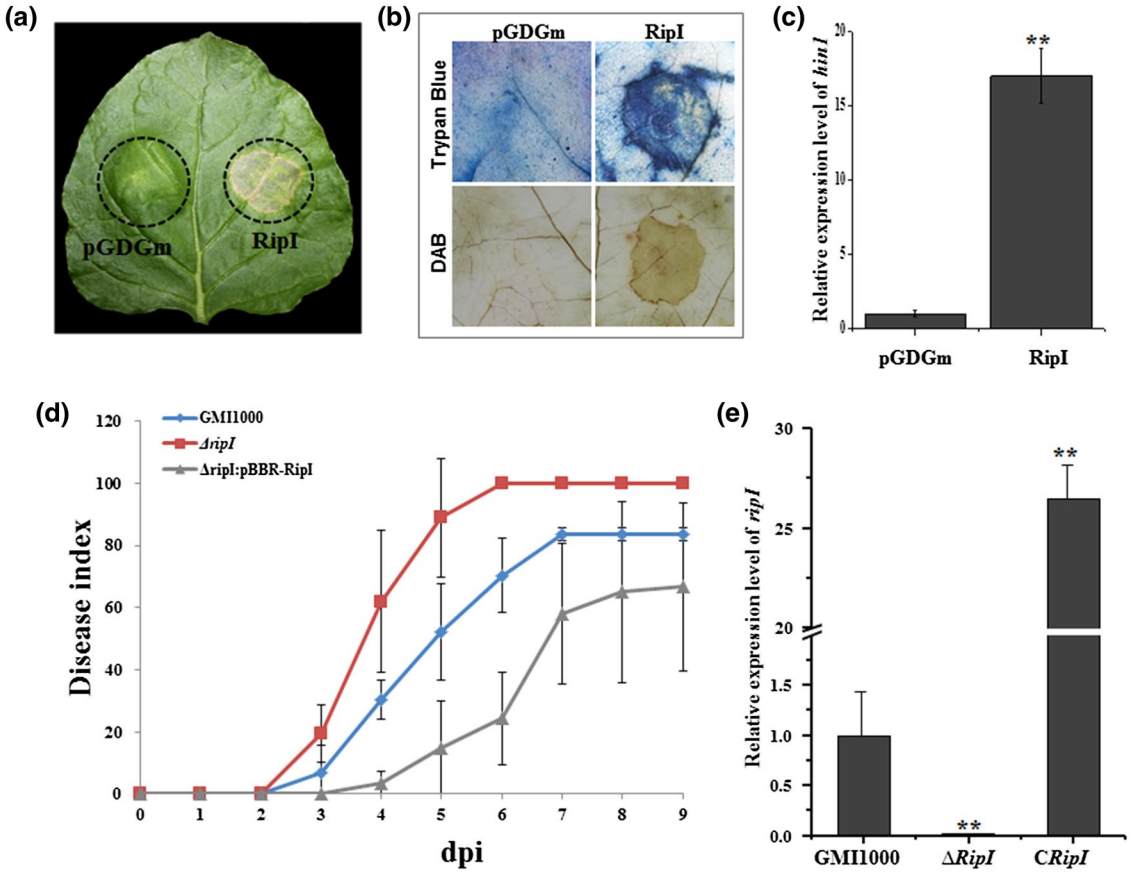

FIGURE 1 RipI induces a defence reaction in host plants. (a) Hypersensitive response (HR) induced by transient heterologous expression of RipI in *Nicotiana benthamiana* leaves. Dotted circles indicate inoculated areas. *Agrobacterium tumefaciens* GV3101 harbouring empty vector control pGDGm or pGDGm-RipI was prepared to OD_{600} = 1.0 and infiltrated into *N. benthamiana* leaves. Photographs were taken at 2 days post-agroinfiltration. (b) Detection of cell death and hydrogen peroxide accumulation by tissue staining. *N. benthamiana* leaves were harvested 36 hr post-agroinfiltration and stained with trypan blue and 3,3′-diaminobenzidine (DAB). (c) Assessment of *hin1* transcript level by quantitative reverse transcription PCR. *hin1* transcript level induced by RipI was compared with that induced by an empty vector pGDGm control (set as 1) at 36 hr post-agroinfiltration. Error bars represent the standard deviation from three replicates. Differences were evaluated using Student's *t* test (**$p < .01$). (d) Progression of bacterial wilt on tomato plants inoculated with the wild type (GMI1000), mutant Δ*ripI*, or the complemented strain Δ*ripI*:pBBR-RipI. Disease severity was rated for 9 days after stem inoculation (dpi). Each time point represents the mean disease severity of six inoculated plants per treatment. Error bars represent the standard deviation from three independent experiments. (e) Transcription of the *ripI* gene in the wild type GMI1000, mutant Δ*ripI*, and the complemented strain Δ*ripI*:pBBR-RipI (C*RipI*). RNAs were extracted from cells cultured in minimal medium M63. Error bars represent the standard deviation from three replicates. Expression level in GMI1000 was set to 1. Differences were evaluated using Student's *t* test (**$p < .01$). All experiments were replicated three times with similar results and representative results are shown

wild type and Δ*ripI* (Figure 1d). We examined the transcript levels of *ripI* in the three strains by quantitative reverse transcription-PCR (RT-qPCR). A higher transcript level was observed in the complemented strain than in the wild type and no transcript was detected in the Δ*ripI* mutant (Figure 1e).

A yeast two-hybrid screen using RipI as bait showed that the *N. benthamiana* transcription factor bHLH93 (*Niben101Scf05329g05016.1*) interacts with RipI (https://solgenomics.net/organism/Nicotiana_benthamiana/genome). To confirm this interaction, recovered pGADT7 plasmid was retransformed into AH109 together with RipI bait. As expected, the transformant was able to grow on SD −His −Leu −Trp −Ade medium supplemented with 20 μg/ml X-α-galactosidase (Figure 2a). Furthermore, a glutathione-S-transferase (GST) pull-down assay confirmed that RipI physically interacts with bHLH93 (Figure 2b).

Next, we examined the subcellular localization of bHLH93 and RipI using *Arabidopsis* protoplasts. Although some protoplasts transfected with RipI-yellow fluorescent protein (YFP) perished, we identified RipI in the nucleus of a number of protoplasts (Figure 2c). This was consistent with the nuclear localization of RipI reported in yeast protoplasts (Deng *et al.*, 2016). bHLH93-YFP was located in the nucleus, membrane, and cytoplasm, and its fluorescence was much brighter than that of RipI-YFP (Figure 2c). Transfection with bHLH93-YFP was more successful than transfection with RipI-YFP, resulting in many more fluorescent protoplasts. Immunoblot analysis using anti-GFP antibodies confirmed the stronger signals from protoplasts transfected with bHLH93-YFP (Figure 2d). No fluorescence was detected from protoplasts co-transfected with RipI and bHLH93.

We investigated the requirement for bHLH93 in HR induction by RipI using tobacco rattle virus (TRV)-induced gene silencing. A 529-bp fragment including coding and 3′ untranslated region (UTR) sequence of *bHLH93* was cloned into pTRV-RNA2, generating

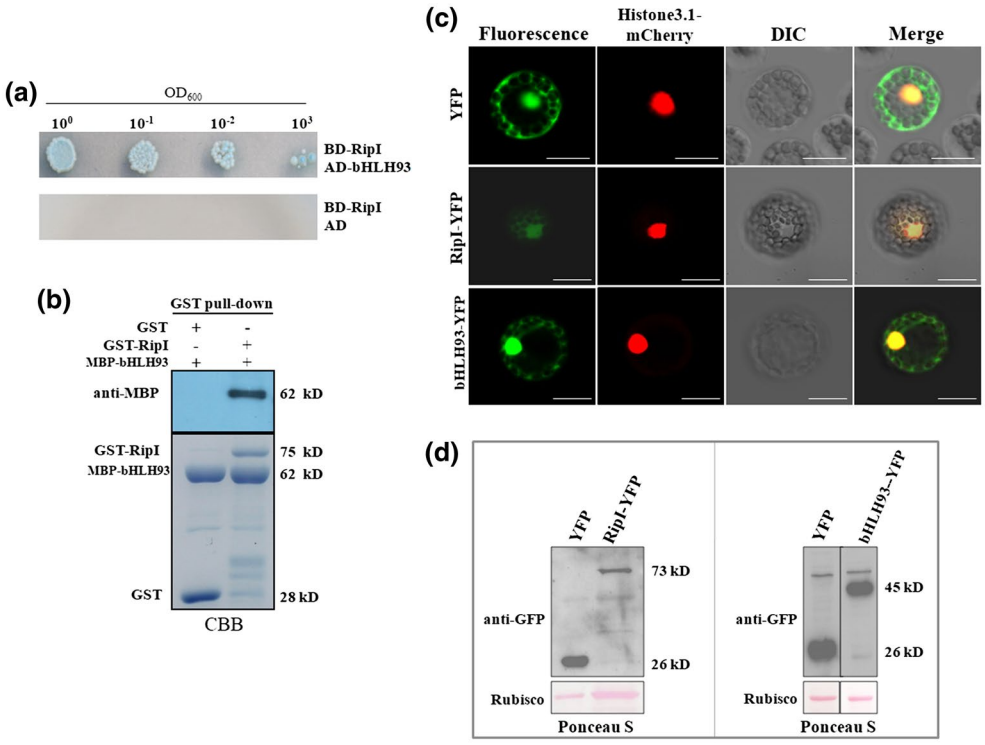

FIGURE 2 RipI interacts with the bHLH93 transcription factor in *Nicotiana benthamiana*. (a) Yeast two-hybrid assays showing the interaction between RipI and bHLH93. The transformants were prepared to a cell density of OD_{600} = 1.0 and diluted in a 10-fold series. For each concentration, 2 μl was spotted and incubated on synthetic defined SD −Ade −Leu −Trp −His plates supplemented with 20 μg/ml X-α-galactosidase (X-α-gal) for 4 days at 30 °C. The transformant containing BD-RipI and empty vector pGADT7 served as a negative control. (b) RipI interacts with bHLH93 in vitro in glutathione-S-transferase (GST) pull-down assays. Recombinant GST-RipI and maltose binding protein (MBP)-bHLH93 fusions were subjected to GST pull-down analysis. GST tag and MBP-bHLH93 fusions were used as the negative control. Gel stained with Coomassie brilliant blue (CBB) is shown. Interacting proteins were identified by immunoblotting using anti-MBP antibodies. The experiment was repeated three times with similar results. (c) Subcellular localization of RipI and bHLH93 in *Arabidopsis* protoplasts. *Arabidopsis* protoplasts were transfected for coexpression of mCherry-histone3.1 (serving as a nuclear localization marker) and either RipI or bHLH93 fused with yellow fluorescent protein (YFP). Images were recorded at 8 hr post-transfection for visualization of YFP fluorescence at 488 nm and mCherry fluorescence at 580 nm. The colour of YFP fluorescence was set to green to facilitate detection of the fluorescence merged with mCherry fluorescence (green merged with red is shown as yellow, while yellow merged with red is shown as orange). Differential interference contrast (DIC) images were also photographed. Bars in all images represent 20 μm. (d) Immunoblot analysis of RipI expression in *Arabidopsis* protoplasts using green fluorescent protein (GFP) polyclonal antiserum (anti-GFP). Total proteins were extracted from *Arabidopsis* protoplasts. *Arabidopsis* protoplasts transfected with empty vector expressing YFP were used as the control. The loading control was RuBisCO stained with Ponceau S. All experiments were replicated three times with similar results and representative results are shown

pTRV2-*bHLH93*. pTRV2-*gfp* carrying the *gfp* (green fluorescent protein) gene fragment served as a negative control and pTRV2-*PDS* (phytoene desaturase) was used to determine silencing efficiency. At 13 dpi, when photobleaching symptoms were observed on the upper leaves of *PDS*-silenced plants, RipI was transiently expressed in *bHLH93*-silenced plants to examine HR induction. RipI agroinfiltrated at three concentrations (OD_{600} = 0.01, 0.1, and 1.0) induced a weaker HR in *bHLH93*-silenced plants than in

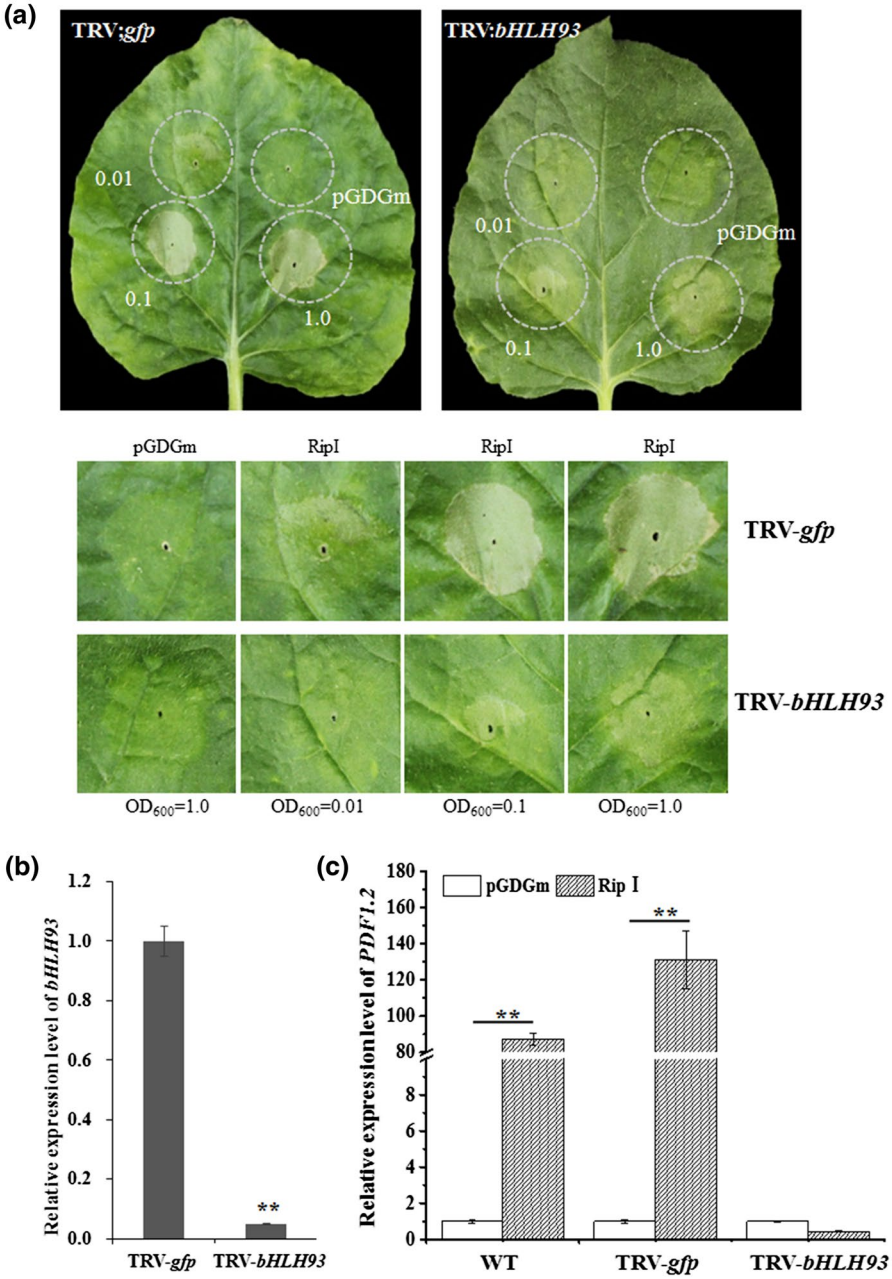

FIGURE 3 Requirement of *bHLH93* for defence induction by RipI. (a) Silencing of *bHLH93* attenuated the hypersensitive response (HR) induced by RipI. Procedures were similar to those shown in Figure 1a. Leaves were inoculated with three concentrations (OD_{600} = 0.01, 0.1, and 1.0) of the pGDGm-RipI construct. The lower images show enlargements of areas exhibiting HR, whereas the whole leaves are shown above. (b) Quantitative reverse transcription PCR analysis of *bHLH93* transcript level in *bHLH93*-silenced plants. RNAs were isolated from the upper new leaves when the photobleaching phenotype was observed in phytoene desaturase (*PDS*)-silenced plants (positive control). The transcript level in plants transformed with tobacco rattle virus (TRV)-*gfp* was used as a control to monitor expression change. Error bars represent the standard deviation from three replicates. Differences were evaluated using Student's *t* tests (**p < .01). (c) bHLH93 is required for *PDF1.2* induction by RipI. *PDF1.2* transcript was quantified in wild type (WT) and in *bHLH93*-silenced plants transiently expressing RipI. The transcript level in plants transformed with TRV-*gfp* served as a negative control. RNAs were isolated 36 hr post-agroinfiltration with RipI. Error bars represent the standard deviation from three replicates. Differences were evaluated using Student's *t* test (**p < .01). All experiments were replicated three times with similar results and representative results are shown

control plants transformed with pTRV1 and pTRV2-*gfp* (TRV-*gfp*) (Figure 3a). RT-qPCR confirmed the silencing effect of *bHLH93* (Figure 3b).

Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) induce diverse but cross-linked defence signalling pathways against microbial pathogens in plants (Zhang *et al*., 2017; Nakano and Mukaihara, 2018). The elicitation of an HR and attenuation of virulence prompted us to investigate whether RipI was associated with any defence signalling pathway. *PDF1.2*, a defensin gene involved in the JA and ET signalling responses (Robert-Seilaniantz *et al*., 2007; Vos *et al*., 2015), was induced by RipI (Figure 3c). However, *PDF1.2* transcript in *bHLH93*-silenced plants was dramatically reduced in response to transient expression of RipI (Figure 3c). The silencing effect of *bHLH93* in *bHLH93*-silenced plants was confirmed by RT-qPCR (Figure S3).

Although RipI induces the defence response, it does not function as an avirulence protein specifying an incompatible interaction with *N. benthamiana* in the GMI1000 genetic background. The *ripI* mutant of GMI1000 retained the ability to induce a HR in *N. benthamiana*, which was jointly determined by RipAA and RipA1 effectors or RipB from strain RS1000 (Poueymiro *et al*., 2009; Nakano and Mukaihara, 2019). The cell death induced by RipI observed here was consistent with the previous report that ectopic expression of RipI leads to apoptosis in yeast (Deng *et al*., 2016). As the arginine at position 117 within the integrase motif is required for its inhibition of yeast growth (Deng *et al*., 2016), the involvement of the integrase motif in inducing cell death in *N. benthamiana* should be clarified.

bHLH proteins belong to a superfamily of transcription factors with redundant members in plants (Ledent and Vervoort, 2001). For example, 133 genes encode bHLHs in *A. thaliana* (Heim *et al*., 2003). These proteins play multiple roles in cell and tissue development and regulate hormone metabolism, improving tolerance to drought stress (Heim *et al*., 2003; Bruex *et al*., 2012). While 324 bHLH family genes in *N. benthamiana* have been identified (Yu *et al*., 2019), the biological roles of most NbbHLH genes remain unknown and only 278 NbbHLHs sequences were released. Though all the NbbHLHs contain bHLH domains, the identities of either full-length sequence or bHLH domain sequence among 278 NbbHLHs are low (Figure S2). The RipI-interacting protein Niben101Scf05329g05016.1 is annotated as bHLH93 in the *N. benthamiana* genome database. In this study, we established that bHLH93 was targeted by the *R. solanacearum* effector RipI and induced a defence reaction in *N. benthamiana*. Both RipI and bHLH93 were located in the nucleus; however, no fluorescence was detected in *Arabidopsis* protoplasts co-transfected with RipI and bHLH93, suggesting that the simultaneous expression of RipI and bHLH93 may accelerate cell death progression, causing a rapid degradation of the nucleus.

JA signalling is a known target of plant bacterial T3SS effectors. The RipAL effector contributes to *R. solanacearum* virulence in pepper (*Capsicum annuum*) plants by inducing JA production (Nakano and Mukaihara, 2018). The *Pseudomonas syringae* effector HopX1 functions as a cysteine protease that activates gene expression in the JA response pathway through degrading jasmonate ZIM domain (JAZ) proteins independently of the Skip-Cullin-F-box (SCF) complex (Gimenez-Ibanez *et al*., 2014). HopZ1a relies on its acetyltransferase activity to acetylate JAZ proteins in advance of proteasome-dependent degradation (Lewis *et al*., 2013). AvrB directly targets a pathway consisting of RAR1, HSP90, MPK4, and RIN4, inducing JA responses and increasing plant susceptibility to *P. syringae* (He *et al*., 2004; Cui *et al*., 2010). Not only that, the gene of *N. benthamiana* related with JA response also was induced by type III secreted effector(s) of *R. solanacearum*, such as the *DS1* gene. *DS1*-silenced plants infected by *R. solanacearum* displayed hyperinduction of *PR4* expression (Nakano *et al*., 2013). The elevated expression of *N. benthamiana PDF1.2* in response to RipI suggests that the JA and ET signalling pathways had been activated. Furthermore, RipI could not induce *PDF1.2* expression in *bHLH93*-silenced plants, implying that bHLH93 functions in signalling pathways mediated by RipI. Further studies are needed to clarify how RipI induces *PDF1.2* expression and whether bHLH93 directly regulates JA and ET signalling.

In conclusion, the *R. solanacearum* effector RipI plays a role in host defence induction, as revealed by the HR induced by transient heterologous expression of RipI in *N. benthamiana*. Mutagenesis of *ripI* in GMI1000 increased its virulence on tomato plants. Furthermore, the bHLH93 transcription factor interacted with RipI and was involved in the RipI-induced defence response in *N. benthamiana*. These findings provide insight into the role of RipI in virulent *R. solanacearum* strains in host plants.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31671988, 31872919, 31701752, and 31801696), the Science Foundation of Fujian Provincial Science & Technology Department (2017J01434), and the Scientific Project of Fujian Provincial Education Department (JA15152). We thank Yule Liu (School of Life Sciences, Tsinghua University, Beijing) for providing the pTRV1, pTRV2, and pTRV2-*PDS* plasmids.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Zhuo T, Wang X, Chen Z, et al. The *Ralstonia solanacearum* effector RipI induces a defence reaction by interacting with the bHLH93 transcription factor in *Nicotiana benthamiana*. *Molecular Plant Pathology*. 2020;21:999–1004. https://doi.org/10.1111/mpp.12937