Short communication

Ralstonia solanacearum* type III secretion system effector Rip36 induces a hypersensitive response in the nonhost wild eggplant *Solanum torvum

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SUMMARY

Ralstonia solanacearum is a Gram-negative soil-borne bacterium that causes bacterial wilt disease in more than 200 plant species, including economically important Solanaceae species. In *R. solanacearum*, the hypersensitive response and pathogenicity (Hrp) type III secretion system is required for both the ability to induce the hypersensitive response (HR) in nonhost plants and pathogenicity in host plants. Recently, 72 effector genes, called *rip* (*Ralstonia* protein injected into plant cells), have been identified in *R. solanacearum* RS1000. RS1002, a spontaneous nalixidic acid-resistant derivative of RS1000, induced strong HR in the nonhost wild eggplant *Solanum torvum* in an Hrp-dependent manner. An *Agrobacterium*-mediated transient expression system revealed that Rip36, a putative Zn-dependent protease effector of *R. solanacearum*, induced HR in *S. torvum*. A mutation in the putative Zn-binding motif (E149A) completely abolished the ability to induce HR. In agreement with this result, the RS1002-derived Δ *rip36* and *rip36*E149A mutants lost the ability to induce HR in *S. torvum*. An E149A mutation had no effect on the translocation of Rip36 into plant cells. These results indicate that Rip36 is an avirulence factor that induces HR in *S. torvum* and that a putative Zn-dependent protease motif is essential for this activity.

vascular parenchyma, proliferates and spreads throughout the vascular system, and produces a large amount of exopolysaccharide (EPS) which blocks water transport, resulting in the wilting and death of infected plants (Genin and Denny, 2012; Schell, 2000). In addition, *R. solanacearum* secretes plant cell wall-degrading enzymes, such as endopolygalacturonase and endoglucanase, which break down pectins and cellulosic glucans in the cell wall (Gonzalez and Allen, 2003; Liu *et al.*, 2005).

Numerous Gram-negative plant pathogenic bacteria use the Hrp type III secretion system (T3SS) encoded by a cluster of approximately 20 hypersensitive response (HR) and pathogenicity (*hrp*) genes (Alfano and Collmer, 1997; Galan and Wolf-Watz, 2006; Tang *et al.*, 2006; Van Gijsegem *et al.*, 2000). Basically, Hrp T3SS acts as a specialized system for the injection of virulence factors, so-called effector proteins, from pathogens into host plant cytoplasm. Generally, effector proteins modulate host cellular functions to establish infection and promote propagation in plants. Recent studies have unveiled biochemical activities of several T3SS effectors, including proteases, phosphatases, ubiquitin ligases, ribosyltransferases, transcriptional activators and phosphothreonine lyases (Dean, 2011; Deslandes and Rivas, 2012; Grant *et al.*, 2006; Hann *et al.*, 2010; Staskawicz *et al.*, 2001; Zhou and Chai, 2008). Like many other plant pathogenic bacteria, Hrp T3SS is essential for the growth of *R. solanacearum* in host plants, as Hrp T3SS-deficient mutants completely lose their pathogenicity, and effector proteins collectively contribute to virulence, as the mutation of a single effector gene produces little or no effect in most cases (Boucher *et al.*, 1987; Cunnac *et al.*, 2004; Kanda *et al.*, 2003; Mukaihara *et al.*, 2004; Zolobowska and Van Gijsegem, 2006). In *R. solanacearum* strain GM1000, 74 T3SS effector proteins have been identified by *in silico* analysis of the whole genome sequence and functional genomic approaches (Cunnac *et al.*, 2004; Poueymiro and Genin, 2009). To date, three effector proteins, namely AvrA, PopP1 and PopP2, have been shown to act as avirulence factors in nonhost plants. For example,

Ralstonia solanacearum is a Gram-negative soil-borne bacterium that causes bacterial wilt disease in more than 200 plant species in over 50 plant families, including many economically important crops, such as potato, tomato, tobacco, banana and eggplant, worldwide (Hayward, 1991; Lebeau *et al.*, 2010). The pathogen enters host plants through natural openings or wounds in the root system, penetrates the xylem vessels, colonizes the root cortex and

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AvrA is responsible for HR elicitation in *Nicotiana tabacum* and *N. benthamiana* (Carney and Denny, 1990; Poueymiro *et al.*, 2009; Robertson *et al.*, 2004). PopP1, a member of the YopJ/AvrRxv (C55) family of cysteine proteases, is the major HR elicitor in *Petunia* St40 (Lavie *et al.*, 2002) and *N. glutinosa* (Poueymiro *et al.*, 2009). PopP2, another member of the YopJ-like family, confers RRS1-R-mediated resistance on *Arabidopsis* (Deslandes *et al.*, 2003). Mutation of the conserved cysteine residue in the catalytic triad of PopP2 abolishes its ability to induce RRS1-R-mediated resistance in *Arabidopsis* (Tasset *et al.*, 2010). Some of the above-mentioned effector proteins have been shown to contribute to virulence in host plants. For example, AvrA is required for the early stage of root infection of the legume *Medicago truncatula* (Turner *et al.*, 2009) and bacterial fitness on tomato leaves (Macho *et al.*, 2010). Macho *et al.* (2010) have also shown that PopP2 is required for bacterial fitness on eggplant or bean. Thus, effector proteins play important roles in the interaction between *R. solanacearum* and its host/nonhost plants.

Plants have evolved the ability to recognize potential pathogens by detecting conserved microbial molecules, such as bacterial flagellin, elongation factor and peptidoglycans, called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), leading to the activation of the basal defence response, called PAMP-triggered immunity (PTI) (Monaghan and Zipfel, 2012). Subsequently, pathogens developed effector proteins to suppress PTI for successful infection. Furthermore, plants developed a system for the recognition of bacterial effectors through the sensing of their virulence by the product of the resistance (*R*) gene. Therefore, effectors are virulence factors which promote disease in compatible interactions, but function as avirulence factors, which induce resistance responses, in incompatible interactions. Understanding the mechanism by which an Avr protein is recognized by the corresponding *R* protein may lead to the identification of the virulence function of the effector protein and the detection system in plants, and contribute to the engineering of more durable resistance in crops.

Fig. 1 Hypersensitive responses (HRs) induced by inoculation of *Ralstonia solanacearum* wild-type (WT) RS1002 and its derivatives in *Solanum torvum* Torubamubiga (A, C) and tobacco (B) leaves. Leaves were infiltrated with bacterial suspensions (OD_{600} of 0.3) of *R. solanacearum* RS1002 (WT), RS1662 ($\Delta rip36$), RS1668 (*rip36E149A*), RS1650 (*avrA::Gm^r*) or RS1273 ($\Delta hrpY$), or the complemented strains RS1672 ($\Delta rip36$ *Tnrip36⁺*) or RS1673 ($\Delta rip36$ *Tnrip36E149A*), and incubated under a 16 h light/8 h dark cycle at 28 °C. Photographs show representative results of *S. torvum* at 1 day post-inoculation (dpi) (A, C) or tobacco at 2 dpi (B) from three independent experiments, which gave similar results. The broken lines represent the infiltrated area. (D) Summary of the plant responses caused by various strains in *S. torvum* and tobacco leaves. NT, not tested.

Fig. 2 Effects of *Agrobacterium*-mediated transient expression of Rip36, Rip36E149A and AvrA in *Solanum torvum* Torubamubiga and tobacco leaves. (A) Construction of a series of pEl2Ω-MCS plasmids expressing the Rip effector. Leaves were infiltrated with *Agrobacterium tumefaciens* GV3101 harbouring no plasmid, an empty plasmid, a plasmid expressing Rip36, Rip36E149A or AvrA at OD₆₀₀ of 0.5, and incubated under a 16 h light/8 h dark cycle at 28 °C. Photographs show representative results of *S. torvum* at 2 days post-inoculation (dpi) (B) or tobacco at 4 dpi (C) from three independent experiments, which gave similar results. The broken lines represent the infiltrated area. (D). Summary of plant responses. HR, hypersensitive response.

In *R. solanacearum* strain RS1000, 72 T3SS effector proteins, the so-called Rip (*Ralstonia* protein injected into plant cells) proteins, have been identified using a calmodulin-dependent adenylate cyclase (Cya) reporter system, and their translocations into plant cells have been demonstrated (Mukaihara *et al.*, 2010). It has been shown that the wild eggplant *Solanum torvum* is highly tolerant to most *R. solanacearum* strains (Clain *et al.*, 2004; Gousset *et al.*, 2005). Hence, this plant species is widely used as a rootstock for eggplant or tomato cultivation. Infiltration of *R. solanacearum* RS1002, a spontaneous nalixidic acid-resistant derivative of RS1000, into the leaves of *S. torvum* Sw. cv. Torubamubiga induced HR, a rapid cell death in the infiltrated area (Fig. 1A). To identify the RS1000 effector protein eliciting HR, we cloned the entire coding region of each *rip* gene into a binary vector pEl2Ω-MCS (Fig. 2A; Ohtsubo *et al.*, 1999) under the control of the high expression promoter 35S-Ω. *Agrobacterium tumefaciens* strain GV3101 was transformed by the resultant plasmids, and then each Rip was transiently expressed in the leaves of *S. torvum* and tobacco (*N. tabacum* L. cv. Xanthi NC) via agroinfiltration. We tested 64 RS1000 effectors and found that Rip36 specifically induced HR in *S. torvum* leaves, but not in tobacco leaves (Fig. 2B–D). Transient expression of AvrA, an avirulence protein in tobacco leaves (Carney

and Denny, 1990; Poueymiro *et al.*, 2009; Robertson *et al.*, 2004), specifically induced HR only in tobacco leaves, indicating that the system worked effectively.

Rip36 is a Zn-dependent protease-like T3SS effector with a putative Zn-binding motif (HExxH). Therefore, we next investigated the molecular mechanism of Rip36-induced HR in *S. torvum*. To examine whether the HExxH motif of Rip36 is required to induce HR, we changed the essential glutamic acid of position 149 to alanine (HEL1H to HAL1H) using a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Experimental details for DNA construction are described in Supporting Information Text S1. Wild-type (WT) *rip36* and *rip36E149A* genes were cloned into a binary vector pEl2Ω-MCS (Ohtsubo *et al.*, 1999) and transiently expressed in leaves of *S. torvum* and tobacco (*N. tabacum* L. cv. Xanthi NC) via a transient *Agrobacterium*-mediated expression system. We found that Rip36E149A no longer induced HR in *S. torvum* (Fig. 2). This result indicates that the putative Zn-dependent protease motif of Rip36 is required for HR elicitation in *S. torvum*.

To confirm the results obtained from the transient expression analysis in *R. solanacearum*, we constructed *rip36* and *avrA* mutant derivatives of strain RS1002, as described in Text S1. The

Table 1 Bacterial strains and plasmids.

Strain	Description	Reference
<i>Ralstonia solanacearum</i>		
RS1002	RS1000 NaI ^r	Mukaihara <i>et al.</i> (2004)
RS1273	RS1002 $\Delta hrpY$	Mukaihara <i>et al.</i> (2004)
RS1650	RS1002 <i>avrA</i> ::Gm ^r	This study
RS1662	RS1002 $\Delta rip36$	This study
RS1668	RS1002 <i>rip36E149A</i>	This study
RS1672	RS1662 chr::mini-Tn5 $rip36^+$	This study
RS1673	RS1662 chr::mini-Tn5 $rip36E149A$	This study
<i>Agrobacterium tumefaciens</i>		
GV3101	Gm ^r , Rif ^r	Lamblin <i>et al.</i> (2001)
<i>Escherichia coli</i>		
DH5 α	F ⁻ λ : ϕ 80d/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA relA1</i>	Takara, Kyoto, Japan
S17-1	<i>thi pro hsdR⁻ hsdM⁺ recA</i> [chr::RP4-2-Tc::Mu-Km::Tn7]	Schäfer <i>et al.</i> (1994)
S17-1 λ pir	λ pir lysogen of S17-1	Simon <i>et al.</i> (1983)
Plasmid		
pEl2 Ω -MCS	Binary vector that can replicate in both <i>E. coli</i> and <i>A. tumefaciens</i> , used for transient expression analysis	Ohtsubo <i>et al.</i> (1999)
pEl2 Ω -MCS- <i>avrA</i>	pEl2 Ω -MCS carrying <i>rip5</i> at <i>XbaI</i> and <i>SpeI</i> sites	This study
pEl2 Ω -MCS- <i>rip36</i>	pEl2 Ω -MCS carrying <i>rip36</i> at <i>XbaI</i> and <i>SpeI</i> sites	This study
pEl2 Ω -MCS- <i>rip36E149A</i>	pEl2 Ω -MCS carrying <i>rip36E149A</i> at <i>XbaI</i> and <i>SpeI</i> sites	This study
pARO-HA-'Cya	CyaA reporter plasmid	Murata <i>et al.</i> (2006)
pARO- <i>rip36</i> -HA-'Cya	pARO-HA-'Cya carrying <i>rip36</i> at <i>XbaI</i> and <i>XhoI</i> sites	Mukaihara <i>et al.</i> (2010)
pK18 <i>mobsacB</i>	Small mobilizable vector, Km ^r , sucrose sensitive (<i>sacB</i>)	Schäfer <i>et al.</i> (1994)
pK18- $\Delta rip36$	pK18 <i>mobsacB</i> -derived plasmid that contains a 1.6-kb chimeric PCR product deleting <i>rip36</i> , Km ^r	This study
pK18- <i>rip36E149A</i>	pK18 <i>mobsacB</i> -derived plasmid that contains a 657-bp <i>rip36E149A</i> , Km ^r	This study
pK18- <i>avrA</i>	pK18 <i>mobsacB</i> -derived plasmid that contains a 2.8-kb PCR product containing <i>avrA</i> , Km ^r	This study
pK18- <i>avrA</i> ::Gm ^r	pK18 <i>mobsacB</i> -derived plasmid that contains disrupted <i>avrA</i> by the insertion of a Gm ^r cassette into pK18- <i>avrA</i> , Gm ^r , Km ^r	This study
pBSL118	Mini-Tn5-derived plasmid vector for insertion mutagenesis, Amp ^r , Km ^r	Alexeyev <i>et al.</i> (1995)
pBSL- <i>rip36</i>	pBSL118-derived plasmid that contains a 2.09-kb <i>rip36</i> , Amp ^r , Km ^r	This study
pBSL- <i>rip36E149A</i>	pBSL118-derived plasmid that contains a 2.09-kb <i>rip36E149A</i> , Amp ^r , Km ^r	This study

R. solanacearum mutants and plasmids used in this study are listed in Table 1.

In *S. torvum*, HR was induced by inoculation of strains RS1002 (parental WT) and RS1650 (*avrA*::Gm^r), but not strains RS1662 ($\Delta rip36$) and RS1668 (*rip36E149A*) (Fig. 1A). HR in tobacco leaves was induced by inoculation of the WT, $\Delta rip36$ and *rip36E149A* strains, but not by the *avrA*::Gm^r strain (Fig. 1B). This result clearly indicates that Rip36 and AvrA of RS1002 are required for HR in *S. torvum* and tobacco, respectively. Furthermore, Rip36 abolished the ability to induce HR in *S. torvum* when the Zn-binding motif HELIH was changed to HALIH, indicating that the putative Zn-dependent protease motif is required for HR elicitation in *S. torvum*. We also confirmed that these HRs were dependent on *hrpY*, a gene encoding an essential component of the Hrp T3SS, supporting the hypothesis that these T3SS effectors determine HR.

To confirm that Rip36 is a crucial avirulence factor in the induction of HR in *S. torvum*, we introduced the WT *rip36* and *rip36E149A* genes into a $\Delta rip36$ mutant strain, and generated two types of complemented strain, as described in Text S1. The leaves of *S. torvum* were infiltrated with the resultant complemented strains, RS1672 ($\Delta rip36$ Tn $rip36^+$) and RS1673 ($\Delta rip36$ Tn $rip36E149A$). As expected, HR in *S. torvum* leaves was restored by the introduction of Tn $rip36^+$, but not Tn $rip36E149A$ (Fig. 1C).

This result further confirmed that Rip36 is an avirulence factor that induces HR in *S. torvum* and that the Zn-protease motif is essential for this HR.

To exclude the possibility that the E149A mutation of Rip36 affects the ability to translocate into plant cells via the Hrp T3SS, we constructed *R. solanacearum* strains expressing the Rip36-HA-'Cya and Rip36E149A-HA-'Cya fusion proteins, as described previously (Murata *et al.*, 2006; Text S1). An *in planta* adenylate cyclase assay was carried out as described previously (Murata *et al.*, 2006). RS1273 ($\Delta hrpY$) was used as a negative control because this strain lacks the ability to translocate effectors into plant cells (Mukaihara and Tamura, 2009). The cyclic adenosine monophosphate (cAMP) in *S. torvum* leaves inoculated with the WT strain expressing Rip36E149A-HA-'Cya was increased to a level similar to that in leaves inoculated with the strain expressing Rip36-HA-'Cya (Fig. 3A). As predicted, the increase in the cAMP level was completely abolished by the $\Delta hrpY$ mutation (Fig. 3A). This result indicates that the E149A mutation in the putative Zn-binding motif has no effect on the translocation of Rip36 into plant cells.

Next, we tested the stability of the Rip36E149A protein by immunoblot analysis. Briefly, *R. solanacearum* strains RS1002 (WT), RS1662 ($\Delta rip36$) and RS1668 (*rip36E149A*) were grown

Fig. 3 Translocation and stability of Rip36 and Rip36E149A. (A) The cyclic adenosine monophosphate (cAMP) levels of *Solanum torvum* leaves inoculated with *Ralstonia solanacearum* RS1002 (*hrp*⁺) or RS1273 (Δ *hrpY*) strains expressing the calmodulin-dependent adenylate cyclase (Cya) fusion with Rip36 or Rip36E149A. The cAMP level is shown as an average of three replications with standard deviations in parentheses. (B) The stability of Rip36 and Rip36E149A in *S. torvum* leaves after infiltration. Leaves of *S. torvum* were infiltrated with RS1002 (lane 1) and the *rip36E149A* (lane 2) and Δ *rip36* (lane 3) mutants, and total proteins were prepared from the inoculated leaves at 15 h post-inoculation. The Rip36 and Rip36E149A proteins were detected by an anti-Rip36 peptide antibody after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Photograph shows a representative result of Western blotting from two independent experiments. An arrow indicates the position of the 20-kDa marker protein.

overnight in BG medium (Bacto-Agar 15 g, glucose 5 g/L) at 28 °C, pelleted, washed with distilled water and then resuspended at an optical density at 600 nm (OD_{600}) of 0.3. The leaves of 5–6-week-old *S. torvum* were infiltrated with the bacterial suspensions and incubated at 28 °C in a growth chamber. After 15 h, the infiltrated parts of the leaves were collected, frozen with liquid nitrogen and ground with a mortar and pestle. Then, 1.5 mL of 50 mM Tris-HCl buffer (pH 7.5) was added per 0.5 mg of leaf tissue. Cell debris was removed by centrifugation twice for 10 min at $10\,000 \times g$, and the supernatant was collected in new tubes. Proteins were then precipitated from the supernatant by adding 5% trichloroacetic acid and incubating for 1 h at 4 °C. Precipitated proteins were pelleted by centrifugation and washed with 70% ethanol, dried, resuspended in 50 μ L PBST buffer (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl, 1.47 mM KH_2PO_4 and Tween-20, pH 7.4) and investigated by Western blot analysis after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Nguyen *et al.*, 2012). A rabbit polyclonal anti-Rip36-specific peptide antibody was generated using the syn-

thetic peptide (peptide sequence: QGLQARSKYKPRNA, 205–218 amino acids) by Operon Biotechnologies (Tokyo, Japan) by the same method as described previously (Nguyen *et al.*, 2012). Bands corresponding to the expected size of the Rip36 protein were detected in WT and *rip36E149A* mutant strains, whereas no band was detected in the Δ *rip36* strain (Fig. 3B). On the basis of these results, it is clear that the point mutation protein Rip36E149A is as stable as WT Rip36, and that the E149A mutation in the Zn-protease motif has no effect on the translocation and stability of Rip36 in plant cells.

To examine whether Rip36 contributes to the virulence of *R. solanacearum* on host eggplant (*Solanum melongena* cv. Senryo-nigou), RS1002 (WT) and the Δ *rip36* and *rip36E149A* mutants [5×10^4 colony-forming units (cfu)/mL] were infiltrated into eggplant leaves and bacterial growth was measured at 2 and 5 days post-inoculation (dpi). This quantitative assay is reported to be more sensitive than the disease scoring method (Macho *et al.*, 2010). However, no significant difference in bacterial multiplication was observed between RS1002, Δ *rip36* and *rip36E149A* mutant strains at either 2 or 5 dpi (Fig. 4A). This result suggests that Rip36 has no or little effect on the virulence of *R. solanacearum* in host eggplant, probably because of the functional redundancy of a large effector repertoire, as reported by Mukaiharu *et al.* (2010).

We further measured the bacterial growth on nonhost *S. torvum*. The leaves of *S. torvum* were infiltrated with RS1002 (WT) and the Δ *rip36* and *rip36E149A* mutants (5×10^4 cfu/mL), and bacterial growth was measured at 2 and 5 dpi. The Δ *rip36* and *rip36E149A* mutants increased significantly in *S. torvum* leaves when compared with the WT RS1002 at both 2 and 5 dpi (Fig. 4B). This indicates that Rip36 is the major avirulence factor in RS1002 that elicits resistance and restricts bacterial growth in *S. torvum* leaves.

In this study, we have shown that the *R. solanacearum* T3SS effector Rip36 functions as an avirulence protein to induce HR in *S. torvum*. We have also shown that the putative Zn-dependent protease motif of Rip36 is essential for its avirulence function. In the fungal pathogen *Magnaporthe grisea*, the causal agent of rice blast disease, an AVR-Pita avirulence protein is known to contain a Zn-dependent protease motif. The protease motif is essential for the avirulence function of AVR-Pita because a point mutation in the conserved motif completely abolishes the ability to induce rice blast resistance mediated by the corresponding disease resistance (R) protein Pi-ta in rice (Orbach *et al.*, 2000). It is also known that AVR-Pita interacts directly with Pi-ta, and that a mutation in the protease motif results in the loss of avirulence and disrupts the physical interaction between them. This direct interaction raises the possibility that the Pi-ta protein may sense the cleavage by AVR-Pita (Jia *et al.*, 2000). Our results also indicate that the Zn-protease activity of Rip36, like Avr-Pita, is required for the induction of HR, although the enzymatic activity has not yet been

Fig. 4 Effect of *rip36* mutation on the growth of *Ralstonia solanacearum* RS1002 on host eggplant (A) and nonhost *Solanum torvum* (B). Leaves were infiltrated with wild-type (WT) RS1002 or $\Delta rip36$ and *rip36E149A* mutants at 5×10^4 colony-forming units (cfu)/mL. Bacterial growth was measured 2 and 5 days after inoculation. Error bars indicate the standard deviation measured from six biological replicates. Means of three independent experiments are presented. Asterisks indicate a significant difference from the growth of the RS1002 WT strain in a *t*-test (****P* < 0.001).

tested. It has been reported that the Rip36 homologue NleD from enteropathogenic *Escherichia coli* cleaves c-JUN N-terminal kinase by its Zn-dependent protease activity and inhibits the inflammatory reaction, an animal innate immunity response (Baruch *et al.*, 2011). In *R. solanacearum*, Rip36 might cleave a particular *S. torvum* protein to exhibit its virulence function, and the resultant peptide fragment might be specifically recognized by a resistance protein, leading to HR. The Rip36 homologue HopH1 from *Pseudomonas syringae* pv. *tomato* DC3000 may contribute to virulence on host plants because the deletion of both *hopH1* and *hopC1* from this pathogen reduced both lesion formation and growth in *Arabidopsis* and tomato (Wei *et al.*, 2007). It is interesting that the putative protease motif of HopH1 is required for its virulence function. The mechanism of HR induction by Rip36 might be similar to that of the cysteine protease effectors AvrRpt2 from

P. syringae pv. *tomato* DC3000 (Axtell and Staskawicz, 2003) and AvrPphB from *P. syringae* pv. *phaseolicola* (Shao *et al.*, 2002). AvrRpt2 and AvrPphB effectors cleave *Arabidopsis* RIN4 and PBS1 proteins, respectively, whose cleavage is monitored by the corresponding R proteins RPS2 and RPS5, respectively (Mackey *et al.*, 2003; Shao *et al.*, 2003). To reveal the mechanism of Rip36-induced HR in *S. torvum*, the identification of the target protein is now under investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Text S1 Plasmid constructions and generation of *Ralstonia solanacearum* strains.