

Short communications

**HopH1 effectors of *Pseudomonas syringae* pv. *tomato* DC3000 and pv. *syringae* B728a induce HR cell death in nonhost eggplant *Solanum torvum***

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**Abstract** (87 words)

HopH1 is an effector protein of *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a and is a homolog of the putative Zn-dependent protease effector Rip36 of *Ralstonia solanacearum*, which induces hypersensitive response (HR) cell death in a nonhost plant, *Solanum torvum* Sw. cv. Torubamubiga. Although *P. syringae* pv. *phaseolicola* (*Pph*) 1448A neither produces HopH1 nor induces HR cell death, *hopH1*-introduced *Pph* 1448A acquired the ability to induce HR. These results indicate that the putative Zn-protease HopH1 effector induces HR cell death in nonhost *S. torvum*.

**Keywords:** Effector; HopH1; HR; Rip36; Zn-protease

*Pseudomonas syringae* is a Gram-negative host-specific pathogen encompassing 50 or more pathovars that cause leaf spot, leaf blight, leaf speck, or bacterial canker disease in the majority of cultivated crops and ornamental plant species all over the world (Agrios 1997). The pathogenicity and ability of this pathogen to cause a hypersensitive response (HR) depend on the Hrp type III secretion system (T3SS), which secretes and translocates effector proteins into the plant cells, thus causing disease in susceptible host plants and triggering HR in resistant host and nonhost plants (Alfano and Collmer 1997).

*P. syringae* T3SS effectors are designated Hrp outer proteins (Hop) and avirulence (Avr) proteins depending on the phenotype by which they were discovered (Lindeberg et al. 2005). Using a comparative genomics and bioinformatics approach based on genome sequences and amino acid features in the N-termini of proteins, *P. syringae* pv. *tomato* (*Pto*) DC3000, *P. syringae* pv. *syringae* (*Psy*) B728a, and *P. syringae* pv. *phaseolicola* (*Pph*) 1448A were predicted to have 58, 29, and 28 effectors, respectively (Greenberg and Vinatzer 2003; Vencato et al. 2006; Lindeberg 2014). To date, the translocation of 40 predicted effectors of *Pto* DC3000 into plant cells was confirmed using a calmodulin-dependent adenylate cyclase reporter system reported by Schechter et al. (2004), and that of 22 effectors of *Psy* B728A was confirmed by assessing the HR-inducing activity of fusion proteins of effector proteins and AvrRpt2 in *Arabidopsis thaliana* possessing the corresponding R protein, RPS2 (Vinatzer et al. 2006). Only 13 effectors are shared between the above three *P. syringae* strains, and the remaining effectors are either unique to one of these strains or shared by only two of them (Baltrus et al. 2011; Lindeberg et al. 2012). These differences in effector repertoires between strains determine the host range of *P. syringae* (Alfano and Collmer 2004).

Among a large repertoire of effectors identified in the *Pseudomonas* genome, a few have been ascribed a role in avirulence (Block et al. 2008). For example, both AvrPphB from *Pph* race 3 and AvrRpt2 from *Pto* T1 are cysteine proteases that cleave the *Arabidopsis* protein kinase PBS1 and RIN4, respectively, and the products of their cleavage are recognized by the respective R proteins RPS5 and RPS2 (Axtell and Staskawicz 2003; Shao et al. 2003) and initiate effector-triggered immunity (ETI). RIN4 degradation induced by AvrPto from *Pto* DC3000 also triggers ETI in tomato and *Nicotiana benthamiana* (Luo et al. 2009). Furthermore, AvrB and AvrRpm1 from *P. syringae* pv. *glycinea* induce phosphorylation of RIN4, which allows it to be recognized by the RPM1 and trigger ETI in *Arabidopsis* (Mackey et al. 2002). The effector HopF1/AvrPphF from *Pph* race 7 shows weak similarity to mono-ADP-ribosyltransferases and is required for avirulence functions in bean and soybean cultivars (Desveaux et al. 2006). The HopZ1a effector from *Psy* A2 has acetyltransferase activity and is perceived by the ZAR1 R protein in *Arabidopsis* (Deslandes and Rivas 2012).

In addition, the HopAS1 effector of *Pto* T1 is recognized by nonhost *Arabidopsis* and *Brassica rapa* (Sohn et al. 2012). Another effector, HopQ1-1 of *Pph* 1448A and *Pto* DC3000, acts as an avirulence determinant in nonhost tobacco plants (Ferrante et al. 2009; Wei et al. 2007), and HopK from *Pto* DC3000 elicited HR in nonhost *N. benthamiana* (Schechter et al. 2004). Effector AvrPtoB from *Psy* B728a elicits nonhost resistance in tomato, which expresses *Pto* family members due to defective E3 ubiquitin ligase activity (Chien et al. 2013). Thus, effectors of *P. syringae* secreted by the T3SS function as a crucial determinant of avirulence in not only resistant host cultivars but also nonhost plants.

We reported that the Rip36 effector in *Ralstonia solanacearum* RS1000 induces HR in nonhost *Solanum torvum* Sw. cv. Torubamubiga (Nahar et al. 2014). *S. torvum* is widely used as a rootstock for eggplant or tomato cultivation and is thought to be an important genetic resource for disease resistance. The Zn-protease motif (HExxH) is conserved in Rip36, and the protease activity seems to be required for the induction of HR because a mutation of this protease motif abolishes the ability to induce HR (Nahar et al. 2014). Whole-genome analysis of *Pto* DC3000, *Psy* B728a, and *Pph* 1448A revealed that *Pto* DC3000 and *Psy* B728a possess a *rip36* homolog, *hopH1*, in which locus tags are Pspto\_0588 and Psyr\_1889, respectively, but *Pph* 1448A does not. Amino acid sequences of both HopH1 from *Pto* DC3000 and *Psy* B728a show very high identity (90%) and similarity (99.5%) to each other and 46% identity and 87.6–89.0% similarity to Rip36. Furthermore, blast search analysis revealed that *Xanthomonas* species also have the HopH1 family. The Zn-protease motif is conserved in all members of the HopH1 family (Fig. 1). In the present study, we investigated whether HopH1 induces HR in *S. torvum*.

We first observed HR between *S. torvum* and the three bacterial strains mentioned above. Different areas of individual leaves of *S. torvum* infiltrated with a bacterial suspension ( $OD_{600} = 0.003$ ) of the wild-type (WT) of *Pto* DC3000, *Psy* B728a, and *Pph* 1448A did not show any changes at 10 days post inoculation (dpi, data not shown). This result suggests that all the bacterial strains tested are avirulent on *S. torvum*. At a higher concentration ( $OD_{600} = 0.3$ ), *Pto* DC3000 and *Psy* B728a induced HR cell death by 1 day after infiltration in leaves of *S. torvum*, but *Pph* 1448A did not (Fig. 2a). These results indicate that HR cell death is a dispensable response for plant defense in these interactions. Mysore and Ryu (2004)

summarized these two types of nonhost resistance as type I and type II. Type I nonhost resistance does not produce any visible change, whereas type II nonhost resistance results in a rapid HR with cell death. The plant response induced after inoculation with *Pph* 1448A coincides with type I nonhost resistance and with *Pto* DC3000 and *Psy* B728a coincides with type II nonhost resistance, although the role of cell death in plant defense needs more investigation.

Because *Pto* DC3000 and *Psy* B728a possess *hopH1* and *Pph* 1448A does not, we examined the ability of HopH1 to induce HR cell death. The *hopH1* genes were amplified using two sets of PCR primers, HopH1-01 and HopH1-02 from *Pto* DC3000 and HopH1-03 and HopH1-04 from *Psy* B728a, respectively (hereafter, all primer sequences are shown in Supplemental Table 1). The resultant 2682-bp and 2240-bp fragments containing 0.7–1.0 kb of the upstream region of the promoter sequence and downstream regions with the entire *hopH1* open reading frame (ORF) were cloned into a pGEM-T Easy vector to produce pGEM-*hopH1*<sub>Pto</sub> and pGEM-*hopH1*<sub>Psy</sub>, respectively (hereafter, all plasmid information is shown in Supplemental Table 2). Then, each DNA insert was transferred into a pBSL118 transposon vector (Alexeyev et al. 1995) to produce pBSL-*hopH1*<sub>Pto</sub> and pBSL-*hopH1*<sub>Psy</sub>. Each plasmid was introduced into the *Pph* 1448A genome through conjugation with *Escherichia coli* S17-1  $\lambda$ pir (Simon et al. 1983) to obtain the *Pph* 1448A transformants *Pph-hopH1*<sub>Pto</sub> and *Pph-hopH1*<sub>Psy</sub>, which possess *hopH1*<sub>Pto</sub> or *hopH1*<sub>Psy</sub>, respectively (hereafter, all bacterial strains used in this study are shown in Table 1). When we infiltrated *S. torvum* leaves with *Pph-hopH1*<sub>Pto</sub> and *Pph-hopH1*<sub>Psy</sub>, we found clear HR (Fig. 2b).

To examine whether HopH1 is indispensable to induce HR cell death on *S. torvum*, we generated *hopH1*-defective mutants in *Pto* DC3000 and *Psy* B728a by conjugation and homologous recombination as previously described (Ichinose et al. 2020). To delete the whole ORF of *hopH1* in *Pto* DC3000 and *Psy* B728a, we carried out inverse PCR using the respective primer (HopH1-05 and HopH1-06 to delete *hopH1*<sub>*Pto*</sub>, and HopH1-07 and HopH1-08 to delete *hopH1*<sub>*Psy*</sub>) and pGEM-*hopH1*<sub>*Pto*</sub> and pGEM-*hopH1*<sub>*Psy*</sub> as the respective templates. Each PCR product was digested with BamHI for  $\Delta$ *hopH1*<sub>*Pto*</sub> and NheI for  $\Delta$ *hopH1*<sub>*Psy*</sub>, then self-ligated to generate the respective *hopH1*-deleted plasmids pGEM- $\Delta$ *hopH1*<sub>*Pto*</sub> and pGEM- $\Delta$ *hopH1*<sub>*Psy*</sub>. The *hopH1*-deleted DNA fragments were excised from the resultant plasmids by EcoRI digestion and introduced into an EcoRI site of the pK18*mob*sacB plasmid (Schäfer et al. 1994). The resulting plasmids, pK18- $\Delta$ *hopH1*<sub>*Pto*</sub> and pK18- $\Delta$ *hopH1*<sub>*Psy*</sub>, were introduced into *Pto* DC3000 and *Psy* B728a by conjugal transfer. Then, the plasmids were excised on a King B agar plate containing 10% sucrose, and deletion of *hopH1* was confirmed by PCR using HopH1-01 and HopH1-02 for *hopH1*<sub>*Pto*</sub> and HopH1-03 and HopH1-04 for *hopH1*<sub>*Psy*</sub>, and by direct sequencing. Infiltration of *S. torvum* leaves with each resultant mutant, *Pto*- $\Delta$ *hopH1* and *Psy*- $\Delta$ *hopH1*, induced typical HR like the WT strains did (Fig. 2a). These results indicate that besides HopH1, *Pto* DC3000 and *Psy* B728a possess another unknown factor that induces HR in *S. torvum*.

We examined whether HopH1 effectors of *Pto* DC3000 and *Psy* B728a also require an HExxH motif for their HR-inducing activity, as we previously found for Rip36 (Nahar et al. 2014). Glutamic acid (E) at position 150 of HopH1<sub>*Pto*</sub> and HopH1<sub>*Psy*</sub> effectors was replaced by alanine (A, E150A) using a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). In brief, using pGEM-*hopH1*<sub>*Pto*</sub> and pGEM-*hopH1*<sub>*Psy*</sub> as templates, two

complementary oligonucleotides containing the mutation (HopH1-09 and HopH1-10 for *hopH1*<sub>PtoE150A</sub> and HopH1-11 and HopH1-12 for *hopH1*<sub>PsyE150A</sub>) were used to mutagenize the Zn-protease HExxH motif according to the manufacturer's protocol. The E150A mutation in the resultant plasmids, pGEM-*hopH1*<sub>PtoE150A</sub> and pGEM-*hopH1*<sub>PsyE150A</sub>, was confirmed by DNA sequencing. In the case of *hopH1*<sub>Pto</sub>, PCR was carried out using pGEM-*hopH1*<sub>PtoE150A</sub> as a template and HopH1-13 and HopH1-14 as primers, and the product was inserted into a pCR-Blunt II TOPO plasmid to generate pCR-*hopH1*<sub>PtoE150A</sub>. The DNA fragments for *hopH1*<sub>PtoE150A</sub> of pCR-*hopH1*<sub>PtoE150A</sub> and *hopH1*<sub>PsyE150A</sub> of pGEM-*hopH1*<sub>PsyE150A</sub> were inserted into the EcoRI site of pBSL118 to produce pBSL-*hopH1*<sub>PtoE150A</sub> and pBSL-*hopH1*<sub>PsyE150A</sub>, respectively. Each plasmid was introduced into the *Pph* 1448A genome through conjugation with *E. coli* S17-1  $\lambda$ pir to generate *Pph-hopH1*<sub>PtoE150A</sub> and *Pph-hopH1*<sub>PsyE150A</sub>, and *S. torvum* leaves were infiltrated with the resultant strains. Unexpectedly, both transformants, *Pph-hopH1*<sub>PtoE150A</sub> and *Pph-hopH1*<sub>PsyE150A</sub>, induced HR on *S. torvum* leaves (Fig. 2b). That is, this mutation (E150A) in the Zn-protease motif did not abolish the activity of HopH1<sub>Pto</sub> and HopH1<sub>Psy</sub> to induce HR cell death on *S. torvum*. This result suggests that the mutation was not sufficient to abolish the protease activity of HopH1 in *P. syringae*; otherwise, protease activity is not required for HopH1-induced HR cell death.

To further examine the necessity of the Zn-protease motif in HR induction, an additional mutation was introduced into the Zn-protease motif in *hopH1*<sub>Pto</sub>. PCR was carried out to generate *hopH1*<sub>PtoE150A•H153A</sub> using pCR-*hopH1*<sub>PtoE150A</sub> as a template and HopH1-15 and HopH1-16 as primers. The resultant plasmid was introduced into *Pph* 1448A by conjugation, and then HR-inducing activity was examined. As shown in Fig. 2b, *Pph-hopH1*<sub>PtoE150A•H153A</sub>

no longer induced HR on *S. torvum*, indicating that the Zn-protease motif is essential for HopH1-induced HR.

In this study, we found that a putative Zn-dependent protease HopH1 from *Pto* DC3000 and *Psy* B728a induced HR on *S. torvum* when heterogeneously expressed in *Pph* 1448A, and the mutation of amino acid residues in the Zn-dependent protease motif (HExxH), E150A and H153A, abolished this induction. The Zn-dependent protease motif is also conserved in the rice blast avirulence factor Avr-Pita of the fungal pathogen *Magnaporthe grisea* and is known to be essential for its avirulence function (Orbach et al. 2000). Avr-Pita directly interacts with Pita, and the putative Avr-Pita metalloprotease may directly cleave Pita (Jia et al. 2000). However, Morel et al. (2018) recently reported that the RipAX2 (Rip36 was renamed RipAX2) of *R. solanacearum* GMI1000 is necessary for resistance in eggplant AG91-25 and the immunity triggered by RipAX2<sub>GMI1000</sub> does not require a putative zinc (Zn)-binding motif. The requirement of protease activity of the HopH1 family might vary among host–bacterial pathogen interactions, or induction of HR cell death might be independent of resistance.

The Rip36 homologue NleD in enteropathogenic *E. coli* is reported to cleave JNK MAP kinase and to inhibit the inflammatory reaction by its Zn-dependent protease activity (Baruch et al. 2011). Mutation of the Zn-protease motif from HExxH to HAxH abolished the activity of both NleD and Rip36. Although, the equivalent mutation of HopH1 did not affect HR-induction, HopH1<sub>*Pto*E150A•H153A</sub> no longer induced HR on *S. torvum*, indicating that protease activity seems to be necessary for HopH1-mediated HR induction. HopH1 might cleave a particular *S. torvum* protein, then the resultant peptide fragment might be specifically recognized by a particular protein to induce HR, or the target protein of HopH1 might be a

negative regulator of a cell death-inducing protein. HopH1 and/or HopC of *Pto* DC3000 might contribute to its virulence in hosts *Arabidopsis* and tomato because inoculation of the *hopH1* and *hopC1* polymutant reduced both lesion formation and growth (Wei et al. 2007), although Rip36 has little or no effect on the virulence in host eggplant because the *rip36* mutant multiplied in the host eggplant like WT (Nahar et al. 2014). Our results indicate that the mechanism of HR induction by HopH1 might be similar to that by Rip36/RipAX2. Identification of the target protein and demonstration of enzymatic activity will provide insight into the HR triggering mechanism of HopH1.

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## Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

## Conflicts of interest

The author has no conflicts of interest to declare.

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## Figure legends

**Fig. 1.** Multiple alignment of Rip36 of *Ralstonia solanacearum* (RsRip36), HopH1 of *Pto* DC3000 and *Psy* B728a and XopG of *Xanthomonas campestris* pv. *campestris* strain B100 (*Xcc*XopG). Identical, strongly similar, and weakly similar amino acids among the four proteins according to ClustalW are represented by asterisks (\*), colons (:), and periods (.),

respectively. The HExxH Zn metalloprotease motif is shown with plus signs above the sequence.

**Fig. 2.** Representative results of test for hypersensitive response at 1 day after inoculation of *Solanum torvum* Torubamubiga leaves with different *Pseudomonas syringae* strains. Leaves were infiltrated with bacterial suspensions (OD<sub>600</sub> of 0.3) of *P. syringae* pv. *tomato* DC3000 wild type (*Pto* WT), its  $\Delta hopHI$  mutant (*Pto*- $\Delta hopHI$ ), pv. *syringae* B728a wild-type (*Psy* WT), its  $\Delta hopHI$  mutant (*Psy*- $\Delta hopHI$ ), and pv. *phaseolicola* 1448A wild type (*Pph* WT) (a) or with pv. *phaseolicola* 1448A possessing *hopHI*<sub>*Pto*</sub>, *hopHI*<sub>*Pto*E150A</sub>, *hopHI*<sub>*Pto*E150A•H153A</sub>, *hopHI*<sub>*Psy*</sub>, *hopHI*<sub>*Psy*E150A</sub>, and its WT strain (b), and incubated under a 16 h light/8 h dark cycle at 28°C. Three independent experiments gave similar results. Dotted lines outline the infiltrated area.

Table 1 Bacterial strains used in this study

| Bacterial strain or plasmid                       | Relevant characteristics                                                                                                                                                                          | Reference or source     |
|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| <i>E. coli</i> strain                             |                                                                                                                                                                                                   |                         |
| DH5 $\alpha$                                      | <i>F'</i> $\lambda$ Ø80 <i>dlacZ</i> Δ <i>M15</i> Δ( <i>lacZYA-argF</i> ) <i>U169 recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>supE44 thi-lgyrA relA1</i> | Takara, Kyoto, Japan    |
| S17-1                                             | <i>thi pro hsdR<sup>-</sup>hsdM<sup>+</sup> recA</i> [chr::RP4-2-Tc::Mu-Km::Tn7]                                                                                                                  | Schäfer et al. (1994)   |
| S17-1 $\lambda$ <i>pir</i>                        | $\lambda$ <i>pir</i> lysogen of S17                                                                                                                                                               | Simon et al. (1983)     |
| <i>P. syringae</i> pv. <i>tomato</i> DC3000       |                                                                                                                                                                                                   |                         |
| <i>Pto</i> WT                                     | DC3000 race 0 wild type, Rif <sup>r</sup>                                                                                                                                                         | Wei et al. (2007)       |
| <i>Pto</i> -Δ <i>hopHI</i>                        | Isolate DC3000 Δ <i>hopHI</i> , Rif <sup>r</sup>                                                                                                                                                  | This study              |
| <i>P. syringae</i> pv. <i>syringae</i> B728a      |                                                                                                                                                                                                   |                         |
| <i>Psy</i> WT                                     | B728a wild type, Rif <sup>r</sup>                                                                                                                                                                 | Loper and Lindow (1987) |
| <i>Psy</i> -Δ <i>hopHI</i>                        | Isolate B728a Δ <i>hopHI</i>                                                                                                                                                                      | This study              |
| <i>P. syringae</i> pv. <i>phaseolicola</i> 1448A  |                                                                                                                                                                                                   |                         |
| <i>Pph</i> WT                                     | 1448A race 6 wild-type                                                                                                                                                                            | Mansfield et al. (1994) |
| <i>Pph-hopHI</i> <sub><i>Pto</i></sub>            | 1448A chr::mini-Tn5 <i>hopHI</i> <sub><i>Pto</i></sub>                                                                                                                                            | This study              |
| <i>Pph-hopHI</i> <sub><i>Pto</i>E150A</sub>       | 1448A chr::mini-Tn5 <i>hopHI</i> <sub><i>Pto</i></sub> E150A                                                                                                                                      | This study              |
| <i>Pph-hopHI</i> <sub><i>Pto</i>E150A•H153A</sub> | 1448A chr::mini-Tn5 <i>hopHI</i> <sub><i>Pto</i></sub> E150A•H153A                                                                                                                                | This study              |
| <i>Pph-hopHI</i> <sub><i>Psy</i></sub>            | 1448A chr::mini-Tn5 <i>hopHI</i> <sub><i>Psy</i></sub>                                                                                                                                            | This study              |
| <i>Pph-hopHI</i> <sub><i>Psy</i>E150A</sub>       | 1448A chr::mini-Tn5 <i>hopHI</i> <sub><i>Psy</i></sub> E150A                                                                                                                                      | This study              |

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PtoHopH1 1 M-ITPSRYPGIYIAPLSNEPTAAHTFKEQAEALDHISAAPSGDKLLRKISTLASQKDRK 58
PsyHopH1 1 M-ITPSRYPGIYIAPLSSEPTAAHTFKEQAEKALDHISAGPSGRELLQEISKLASKKDRK 59
RsRip36 1 M-LIQTQYPGFYIATASDNQREADRYAGKVNEALGKIASGRSGDELLRGISSLSATRQRK 59
XccXopG 1 MPISQTNFPGIYISTQTSAREEP--YKNQVESALEKIAAGSSGSALLQGLSAISARKNRK 58
* : :.:*:*:*:. :. . : :.:.* * :*:. ** ** : * :.: :.*

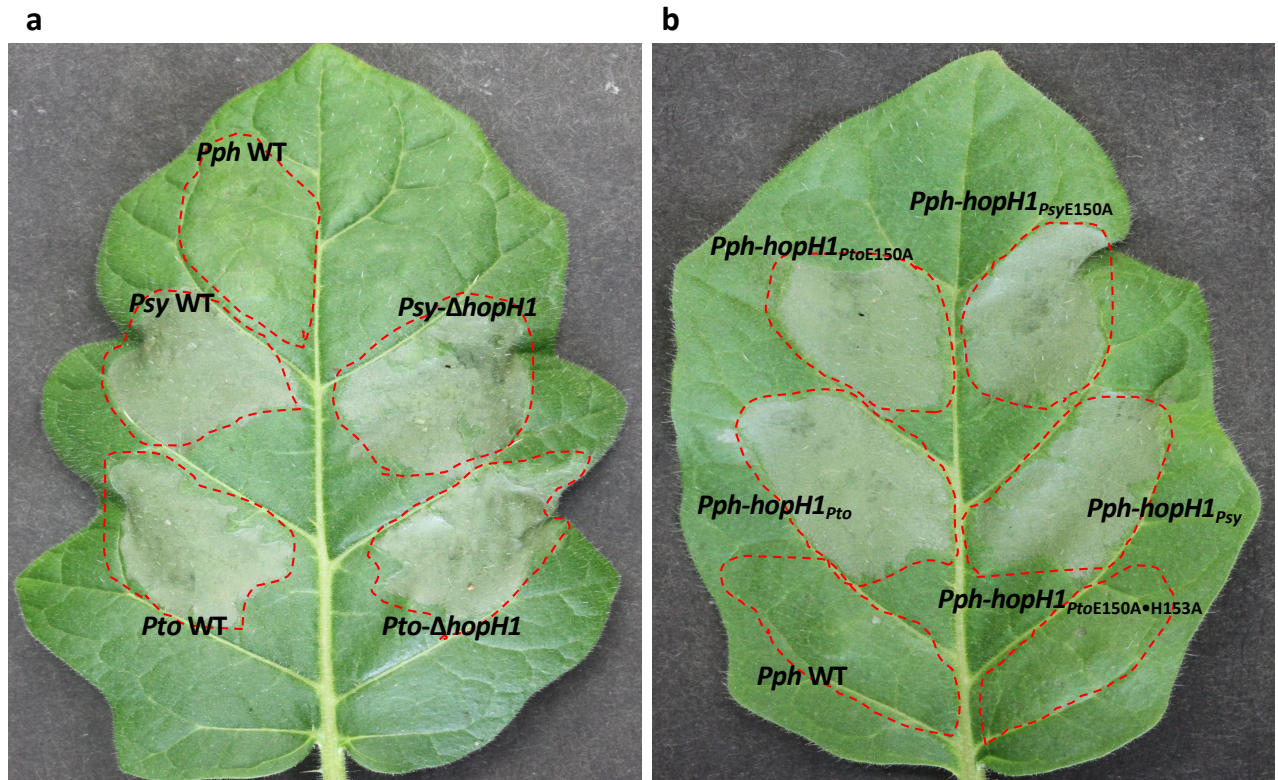
PtoHopH1 59 VTLKEIEINNQCYTEAVLSRRQLEKYEPENFNENRHIAQLSRKGTFTKGECSNAIIGWS 119
PsyHopH1 60 VTLKEIEINNQCYTDAVLSRRQLEKYEPEDFNENRRIASRLSRKGTFTKGECSNAIIGWS 119
RsRip36 60 LTISEIDSDDDPGTEAVLTRPQIAAYEPSDFRANKRIAKQFARGEVSSEPAGCSAIVNWN 119
XccXopG 59 VTIAEIGAEAQPNTRAVLSASEVEKYDPETFADNLELAKERAR-----KGKGCNAIIIEWS 113
*: * : : * ***: : : * :. * * . : * : : * : * : * :

+++++
PtoHopH1 120 PDKASIRLNQNGSPLHLGMDNDDKITTLAHEL VHARHVLGGSSLAD--GGDRYNPRTGSGK 178
PsyHopH1 120 PDKASIRLNQNGSPLRLGMDNDDKITTLAHEL VHVRHVLGGSSLAD--DGDYRNPTGSGK 178
RsRip36 120 P-KTSIKLSRNGSPKRLHKDPKESFAVLAHEL I HARHVMAGTSKAW--SGDRYNETSEAGQ 177
XccXopG 114 P-QSHIELNSNGSPLRLGSDPEESFVLAHEL I HAQHLLAGTSRAYKGGDRYDETSEAGK 172
* : : *. *. **** : * * . : . . . . . ***** : * : * : * : * : * : * : * :

PtoHopH1 179 EELRAVGLDKYRYSLTKKPSENSIRA EHGLPLRMKYRAHQ 218
PsyHopH1 179 EELRAVGLDKYSYSLTKEPSENSIRA EHGLPLRMKYRPHQ 218
RsRip36 178 EELRAVGLGAYAHAYTGEPTENSIRA EQGLQARSKYKPRNA 218
XccXopG 173 EELRAVGVGKYEYRKTRQPSENSIRQE HGLPVRKKYKPHGM 213
***** : . * : * : * : ***** * : * * * * : * : :

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**Fig.1.** Multiple Alignment of Rip36 of *R. solanacearum* (*RsRip36*), HopH1 of *Pto* DC3000 and *Psy* B728a and XopG of *Xanthomonas campestris* pv. *campestris* strain B100 (*XccXopG*). The identical, strongly similar and weakly similar amino acids among four proteins are represented by asterisks (\*), colon (:) and periods (.), respectively according to ClustalW. The HExxH Zn metalloprotease motif is shown with plus signals above the sequence.



**Fig. 2.** Hypersensitive response induced by inoculation of different *Pseudomonas syringae* strains. *Solanum torvum* Torubamubiga leaves were infiltrated with bacterial suspensions ( $OD_{600}$  of 0.3) of *P. syringae* pv. *tomato* DC3000 wild type (*Pto* WT), its  $\Delta hopH1$  mutant (*Pto-ΔhopH1*), pv. *syringae* B728a wild-type (*Psy* WT), its  $\Delta hopH1$  mutant (*Psy-ΔhopH1*), pv. *phaseolicola* wild type (*Pph* WT) (a), pv. *phaseolicola* 1448A possessing *hopH1<sub>Pto</sub>* (*Pph-hopH1<sub>Pto</sub>*), *hopH1<sub>PtoE150A</sub>*, *hopH1<sub>PtoE150A-H153A</sub>*, *hopH1<sub>Psy</sub>*, *hopH1<sub>PsyE150A</sub>*, and its wild-type strain (b), and incubated under a 16 h light/8 h dark cycle at 28° C. Photographs show representative results of *S. torvum* at 1 dpi from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area.

Supplemental Table 1. Primers used in PCR and site-directed mutagenesis

| Primer name | Sequence (5'-3')                                        | Purpose                                               |
|-------------|---------------------------------------------------------|-------------------------------------------------------|
| HopH1-01    | CTTCCGCAAACCCCGGAGTA                                    | Amplification of <i>hopH1</i> <sub>Pto</sub>          |
| HopH1-02    | CCCCATGAGGTGGTACACGA                                    | Amplification of <i>hopH1</i> <sub>Pto</sub>          |
| HopH1-03    | CCCGAATTCTGTGGGCGCTC                                    | Amplification of <i>hopH1</i> <sub>Psy</sub>          |
| HopH1-04    | TGGCGAATTCGACCTTGCAC                                    | Amplification of <i>hopH1</i> <sub>Psy</sub>          |
| HopH1-05    | <u>cgggatcc</u> AGTTATCCTGAAAGCTAATTGAGTTGATC           | Deletion of <i>hopH1</i> <sub>Pto</sub>               |
| HopH1-06    | <u>cgggatcc</u> GGCAACCTACTGTAAGTTATTTTTTAGCG           | Deletion of <i>hopH1</i> <sub>Pto</sub>               |
| HopH1-07    | <u>ctagctagc</u> GAAATTGCAACTATTGG                      | Deletion of <i>hopH1</i> <sub>Psy</sub>               |
| HopH1-08    | <u>ctagctagc</u> AGGCCACATCAATAGGACAA                   | Deletion of <i>hopH1</i> <sub>Psy</sub>               |
| HopH1-09    | CACGACCCTAGCTCATG <u>cgc</u> CTCGTTCATGCTCGAC           | Generation of <i>hopH1</i> <sub>PtoE150A</sub>        |
| HopH1-10    | GTCGAGCATGAACGAGC <u>gc</u> CATGAGCTAGGGTCGTG           | Generation of <i>hopH1</i> <sub>PtoE150A</sub>        |
| HopH1-11    | CACGACCTTGGCTCATG <u>cgc</u> CTAGTTCATGTTCGAC           | Generation of <i>hopH1</i> <sub>PsyE150A</sub>        |
| HopH1-12    | GTCGAACATGAACTAGC <u>gc</u> CATGAGCCAAGGTCGTG           | Generation of <i>hopH1</i> <sub>PsyE150A</sub>        |
| HopH1-13    | TTTGCGCATCTGCGCATCGA                                    | Construction of pCR- <i>hopH1</i> <sub>PtoE150A</sub> |
| HopH1-14    | AATCATGGGCGTCCTAATTCGG                                  | Construction of pCR- <i>hopH1</i> <sub>PtoE150A</sub> |
| HopH1-15    | AGCTCATG <u>cgc</u> CTCGTT <u>gct</u> CGCTCGACATGTGTTAG | Generation of <i>hopH1</i> <sub>PtoE150A•H153A</sub>  |
| HopH1-16    | CTAACACATGTCGAGCA <u>gc</u> AACGAGC <u>gc</u> CATGAGCT  | Generation of <i>hopH1</i> <sub>PtoE150A•H153A</sub>  |

Lower-case letters indicate artificial nucleotides. Underlined sequences in HopH1-05 to -08 indicate BamHI- or NheI-recognition sequences. Underlines in HopH1-09 to -12 and HopH1-15 to -16 indicate codons substituted by site-directed mutagenesis. Lower-case, bold letters are substituted nucleotides.

Supplementary Table 2. Plasmid DNA used in this study

| Plasmid                                      | Description                                                                                                          | Reference or source          |
|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------|------------------------------|
| pGEM-T Easy                                  | Cloning vector, Amp <sup>r</sup>                                                                                     | Promega, Madison, WI, USA    |
| pGEM- <i>hopHI</i> <sub>Pto</sub>            | pGEM-T Easy carrying PCR product of <i>hopHI</i> <sub>Pto</sub> (2682-bp), Amp <sup>r</sup>                          | This study                   |
| pGEM- <i>hopHI</i> <sub>PtoE150A</sub>       | pGEM- <i>hopHI</i> <sub>Pto</sub> -derived plasmid that contains <i>hopHI</i> <sub>PtoE150A</sub> , Amp <sup>r</sup> | This study                   |
| pGEM-Δ <i>hopHI</i> <sub>Pto</sub>           | Whole <i>hopHI</i> <sub>Pto</sub> -deleted pGEM- <i>hopHI</i> <sub>Pto</sub> , Amp <sup>r</sup>                      | This study                   |
| pGEM- <i>hopHI</i> <sub>Psy</sub>            | pGEM-T Easy carrying PCR product of <i>hopHI</i> <sub>Psy</sub> (2240-bp), Amp <sup>r</sup>                          | This study                   |
| pGEM- <i>hopHI</i> <sub>PsyE150A</sub>       | pGEM- <i>hopHI</i> <sub>Psy</sub> -derived plasmid that contains <i>hopHI</i> <sub>PsyE150A</sub> , Amp <sup>r</sup> | This study                   |
| pGEM-Δ <i>hopHI</i> <sub>Psy</sub>           | Whole <i>hopHI</i> <sub>Psy</sub> -deleted pGEM- <i>hopHI</i> <sub>Psy</sub> , Amp <sup>r</sup>                      | This study                   |
| pK18 <i>mobsacB</i>                          | Small mobilizable vector, sucrose-sensitive ( <i>sacB</i> ), Km <sup>r</sup>                                         | Schäfer et al. (1994)        |
| pK18-Δ <i>hopHI</i> <sub>Pto</sub>           | pK18 <i>mobsacB</i> containing 2.0-kb chimeric PCR product deleting <i>hopHI</i> <sub>Pto</sub> , Km <sup>r</sup>    | This study                   |
| pK18-Δ <i>hopHI</i> <sub>Psy</sub>           | pK18 <i>mobsacB</i> containing 1.6-kb chimeric PCR product deleting <i>hopHI</i> <sub>Psy</sub> , Km <sup>r</sup>    | This study                   |
| pCR-Blunt II TOPO                            | Cloning vector for PCR product, Km <sup>r</sup>                                                                      | Invitrogen, Waltham, MA, USA |
| pCR- <i>hopHI</i> <sub>PtoE150A</sub>        | pCR-Blunt II-TOPO carrying PCR product of <i>hopHI</i> <sub>PtoE150A</sub> (1260-bp), Km <sup>r</sup>                | This study                   |
| pCR- <i>hopHI</i> <sub>PtoE150A•H153A</sub>  | pCR-Blunt II-TOPO carrying <i>hopHI</i> <sub>PtoE150A•H153A</sub> (1260-bp), Km <sup>r</sup>                         | This study                   |
| pBSL118                                      | Mini-Tn5-derived plasmid vector for insertion mutagenesis, Amp <sup>r</sup> , Km <sup>r</sup>                        | Alexeyev et al. (1995)       |
| pBSL- <i>hopHI</i> <sub>Pto</sub>            | pBSL118 carrying <i>hopHI</i> <sub>Pto</sub> (2682-bp), Amp <sup>r</sup> , Km <sup>r</sup>                           | This study                   |
| pBSL- <i>hopHI</i> <sub>PtoE150A</sub>       | pBSL118 carrying <i>hopHI</i> <sub>PtoE150A</sub> (1260-bp), Amp <sup>r</sup> , Km <sup>r</sup>                      | This study                   |
| pBSL- <i>hopHI</i> <sub>PtoE150A•H153A</sub> | pBSL118 carrying <i>hopHI</i> <sub>PtoE150A•H153A</sub> (1260-bp), Amp <sup>r</sup> , Km <sup>r</sup>                | This study                   |
| pBSL- <i>hopHI</i> <sub>Psy</sub>            | pBSL118 carrying <i>hopHI</i> <sub>Psy</sub> (2240-bp), Amp <sup>r</sup> , Km <sup>r</sup>                           | This study                   |
| pBSL- <i>hopHI</i> <sub>PsyE150A</sub>       | pBSL118 carrying <i>hopHI</i> <sub>PsyE150A</sub> (2240-bp), Amp <sup>r</sup> , Km <sup>r</sup>                      | This study                   |

Amp<sup>r</sup> ampicillin resistance, Km<sup>r</sup> kanamycin resistance, Nal<sup>r</sup> nalidixic acid resistance