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Short communication

Degeneration of *hrpZ* gene in *Pseudomonas syringae* pv. *tabaci* to evade tobacco defence: an arms race between tobacco and its bacterial pathogen

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SUMMARY

The HrpZ harpin of *Pseudomonas syringae* is known to induce a hypersensitive response (HR) in some plants. In *P. syringae* pv. *tabaci* (*Pta*), the harpin gene *hrpZ* has been spontaneously disrupted by an internal deletion in its open reading frame and a frame shift. The loss of the ability of the recombinant harpin polypeptide of *Pta* to induce HR despite the high sensitivity of tobacco plants to harpin led us to investigate the meaning of the disrupted *hrpZ* gene in the virulence of *Pta* 6605. The *hrpZ* gene from *P. syringae* pv. *pisi* was introduced into wild-type (WT) *Pta*. The *hrpZ*-complemented *Pta* secreted harpin into the culture medium, but failed to cause disease symptoms by both infiltration and spray inoculation. Inoculation with the *hrpZ*-complemented *Pta* induced defence responses in tobacco plants, whereas the defence responses of tobacco plants were not prominent on inoculation with WT *Pta*. These results indicate that an ancestor of *Pta* might have disrupted *hrpZ* by an internal deletion to evade plant defences and confer the ability to cause disease in tobacco plants.

The genetic region that causes the hypersensitive response (HR) in nonhost plants and pathogenicity in host plants was first identified in *Pseudomonas syringae* pv. *phaseolicola* (*Pph*) and designated as the *hrp* gene cluster (Lindgren *et al*., 1986). *Hrp* genes, which are found in many Gram-negative phytopathogenic bacteria, encode a type III secretion apparatus, regulatory proteins and accessory proteins (Jin *et al*., 2003; Mansfield, 2009). Bacteria inject Hrp outer proteins (Hops) directly into the plant cytoplasm and release harpin proteins from the bacterial cells through the type III secretion system (T3SS) (Alfano and Collmer, 2004; Grant *et al*., 2006). Unlike other Hops, harpins elicit HR from outside the cells. Harpins are known to be rich in glycine, lacking in cysteine and to have heat-stable HR-inducing activity, and thus they are thought to possess one of the pathogen-associated molecular patterns (PAMPs) (He *et al*., 1993). We have cloned the harpin genes *hrpZ* from *P. syringae* pathovars *pisi* (*Ppi*), *glycinea* (*Pgl*) and *tabaci* (*Pta*), and found that the deduced amino acid sequences of these harpin proteins are well conserved. However, *hrpZ* in *Pta* 6605 has a 326-bp internal deletion in the central region (which corresponds to amino acids 131–239 in the highly homologous harpin from *Pgl*) of the open reading frame, which results in a significant frame shift (Taguchi *et al*., 2001). Because all isolates of *Pta* investigated so far retain the deletion, this genetic feature might be specific to pv. *tabaci*. These results indicate that the *hrpZ* gene was evolutionarily disrupted in *Pta*. To evaluate the elicitor activity of the truncated harpin protein from *Pta*, a recombinant harpin from *Pta* ($harpin_{Pta}$) possessing only 130 (N)-terminal amino acids with 10 unrelated amino acids in the carboxyl terminal extension was expressed in *Escherichia coli*. The purified recombinant $harpin_{Pta}$ did not induce HR in tobacco plants (Taguchi *et al*., 2001). $Harpin_{Pta}$ was not only unable to induce defence responses, but *Pta* 6605 did not produce $harpin_{Pta}$ in the intracellular fraction or secrete it into the extracellular space (Taguchi *et al*., 2001). Although the role of harpin protein in pathogenesis is not yet clear, mutational analysis of *hrpZ* in *P. syringae* pv. *tomato* (*Pto*) indicated that $harpin_{Pto}$ is not a prerequisite for virulence against the host tomato plant (Charkowski *et al*., 1998). In this study, an *hrpZ*-complemented strain of *Pta* 6605 was created, and the reduction or

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enhancement of its virulence was analysed in comparison with the wild-type (WT) strain. Furthermore, evolutionary aspects of plant-pathogenic bacteria are discussed.

The *hrpZ* operon consists of *hrpA*, *hrpZ*, *hrpB*, *hrcJ*, *hrpD* and *hrpE* (He, 1996). To complement the *hrpZ* gene in the WT strain, a set of primers (pro-F, 5′-*cgGAATTC*gagctcgatatcccacgtcg-3′; hrpZ-R, 5′-*cgGAATTC*tcaggctgcagcctgattgc-3′; italic letters are noncomplementary nucleotides and capital letters indicate an *Eco*RI site) was first used to amplify a DNA fragment containing *hrpA*, *hrpZ* and the promoter of the *hrpZ* operon using a cosmid clone that possesses the entire *hrpZ* operon of *Ppi* as the template DNA (Nakada *et al*., 1999) by polymerase chain reaction (PCR). The PCR product was digested with *Eco*RI and inserted into an *Eco*RI-linearized broad-host-range plasmid vector pDSK519 (Keen *et al*., 1988) to construct p*hrpAZ*. To reveal the effect of exogenous *hrpA* in the *hrpAZ*-complemented strain, we also generated complemented strains with the plasmid containing only *hrpA* or only *hrpZ*. Three sets of PCR primers [pro-F and pro-R (5′-*cgGGATCC*accgatcgtgttgacgacac-3′) for the *hrpZ* promoter; pro-F and hrpA-R (5′-tcagaactggacgaccgagt) for the *hrpZ* promoter with the *hrpA* coding region; and hrpZ-F (5′-*cgGGATCC*atgcagagtctcagtattaa-3′) and hrpZ-R for the *hrpZ* coding region; italic letters are noncomplementary nucleotides and capital letters indicate a *Bam*HI site] were used to amplify the respective DNA fragment using a p*hrpAZ* plasmid as a template. PCR products of the *hrpZ* promoter and *hrpZ* coding region were ligated after digestion with *Bam*HI. The resulting fragment was subjected to PCR again using primers pro-F and hrpZ-R, and introduced into a pDSK519 vector to construct p*hrpZ*. The DNA fragment of the *hrpZ* promoter with *hrpA* was also inserted into a pDSK519 vector to construct p*hrpA*. The plasmids p*hrpA*, p*hrpZ* and p*hrpAZ*, and an empty pDSK519 vector as a control, were introduced into the *Pta* 6605 WT strain by conjugation via *E. coli* S-17 using a method described previously (Shimizu *et al*., 2003).

To confirm the production of harpin protein, WT *Pta* and *hrpAZ*-complemented strains were incubated for 24 h in nutrient-poor MMMF (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM mannitol, 10 mM fructose, pH 5.7) medium, and each bacterial culture supernatant was concentrated. As shown in Fig. 1, *Pta* 6605 WT did not produce harpin either inside or outside the bacterium. However, the *hrpAZ*-complemented strain produced harpin protein both inside and outside the cells. p*hrpAZ* was also introduced into the T3SS-defective mutant Δ*hrcC* strain in *Pta* 6605 (Marutani *et al*., 2005). The *hrpAZ*-complemented Δ*hrcC* strain produced harpin protein inside the cells. However, harpin protein was not detected in the culture supernatant, suggesting that the *hrpAZ*-complemented Δ*hrcC* strain failed to secrete harpin protein outside the bacterium via the defective T3SS. *HrpA* encodes a major structural protein of the Hrp pilus (Roine *et al*., 1997). Although the *hrpA*-complemented strain did not produce

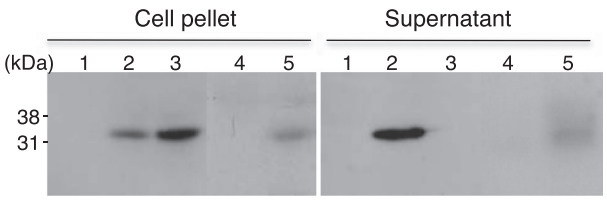

Fig. 1 Immunoblot analysis of harpin from each strain of *Pseudomonas syringae* pv. *tabaci* (*Pta*). Each strain was grown in modified KB (2% proteose peptone No. 3, 1.6 nM $MgSO_4 \cdot 7H_2O$, 8.6 mM K_2HPO_4, 1% glycerol) medium for 24 h and incubated in MMMF (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM mannitol, 10 mM fructose, pH 5.7) medium for 24 h. The bacterial culture was centrifuged, and the supernatant was concentrated 100-fold. Harpin protein in the cell pellet and supernatant was detected as described previously by Taguchi *et al*. (2001). 1, *Pta* (wild-type, WT); 2, *Pta* (p*hrpAZ*); 3, *Pta* Δ*hrcC* (p*hrpAZ*); 4, *Pta* (p*hrpA*); 5, *Pta* (p*hrpZ*).

or secrete harpin protein, the *hrpZ*-complemented strain did. However, the secretion of harpin by the *hrpZ*-complemented strain was less than that by the *hrpAZ*-complemented strain. This result indicates that exogenous expression of *hrpA* facilitates the secretion of harpin.

To investigate the effect of *hrpAZ* complementation in *Pta* 6605 on tobacco plants, tobacco leaves were inoculated with *Pta* 6605 WT and the *hrpAZ*-complemented strain. The *Pta* WT strain caused typical and severe wildfire disease in tobacco, whereas the *hrpAZ*-complemented strain did not cause any disease symptoms using infiltration and dip inoculation methods (Fig. 2A). Consistent with the inoculation of the *hrpAZ*-complemented *Pta*, inoculation of the solo *hrpZ*-complemented *Pta* did not cause severe disease symptoms; however, *Pta* WT with the solo *hrpA*-complemented *Pta* and the *Pta*-possessing empty plasmid produced typical symptoms of wildfire disease by both infiltration and dip inoculation methods, although the disease symptoms caused by the inoculation of the solo *hrpA*-complemented *Pta* were not as severe as those caused by the *Pta* WT strain (Fig. 2A). These results indicate that *hrpZ*-complemented *Pta* shows reduced virulence as a result of the expression of exogenous *hrpZ*, and that the exogenous expression of *hrpA* also reduces the virulence slightly. Furthermore, bacterial propagation of *hrpAZ*-, *hrpZ*- and *hrpA*-complemented strains was reduced compared with that of the *Pta* WT strain and empty plasmid possessing *Pta* (Fig. 2B).

Because tobacco plants are highly sensitive to harpin elicitors and mount strong defence responses, including hypersensitive cell death, oxidative burst and the activation of defence-related genes (Andi *et al*., 2001; He *et al*., 1993; Ichinose *et al*., 2001), the reduction of virulence in the *hrpAZ*-complemented strain might be a consequence of the induction of harpin-triggered defence responses. To examine tobacco defence responses, callose deposition and harpin-induced gene expression were

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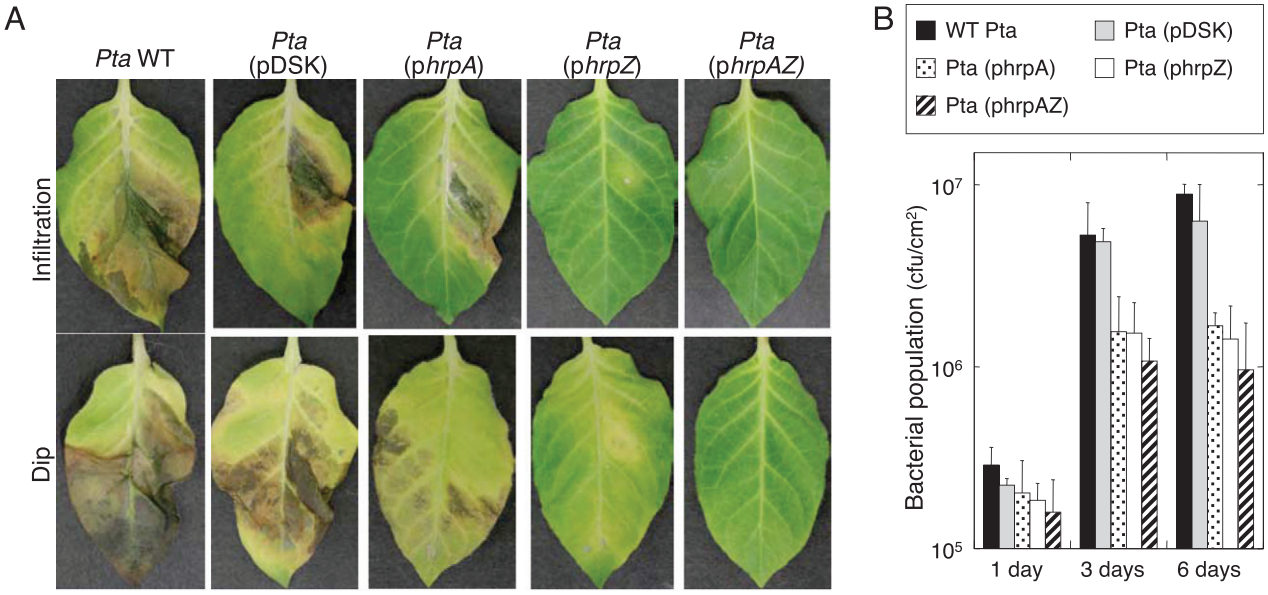

Fig. 2 Inoculation of tobacco leaves with *Pseudomonas syringae* pv. *tabaci* (*Pta*) wild-type (WT), *hrpA*-, *hrpZ*- and *hrpAZ*-complemented strains. (A) Tobacco leaves (*Nicotiana tabacum* L. cv. Xanthi NC) were inoculated by infiltration with 2×10^5 colony-forming units (cfu)/mL (top) and by the dip inoculation method with 2×10^8 cfu/mL (bottom), and were incubated at 23°C for 10 days. (B) The bacterial population of each strain on host tobacco leaves was measured at 1, 3 and 6 days after dip inoculation. Bacterial strains were *Pta* 6605 WT (*Pta* WT) and an empty vector possessing *Pta* [*Pta* (pDSK)], *hrpA* complement [*Pta* (p*hrpA*)], *hrpZ* complement [*Pta* (p*hrpZ*)] and *hrpAZ* complement [*Pta* (p*hrpAZ*)]. The bars represent standard deviations for at least three independent experiments.

investigated. Inoculation of the *hrpAZ*-complemented strain induced callose deposition in tobacco leaves, whereas inoculation of *Pta* WT or empty plasmid possessing *Pta* did not (Fig. 3A). *Pta* (p*hrpA*) weakly induced callose deposition, and the induction of callose deposition by *Pta* (p*hrpZ*) was intermediate between that of *Pta* (p*hrpA*) and *Pta* (p*hrpAZ*). Semi-quantitative reverse transcription (RT)-PCR analysis was performed to monitor the expression of the harpin-induced 1 gene (*HIN1*; Gopalan *et al*., 1996). *HIN1* is a typical plant defence gene, and tobacco *HIN1* mRNA is accumulated as a result of treatment with harpins from *Erwinia amylovora* Ea321 and *P. syringae* pv. *syringae* (*Pss*) 61 (Gopalan *et al*., 1996). Total RNA was purified from tobacco leaves that had been inoculated with *Pta* 6605 WT, *hrpA*-, *hrpZ*- or *hrpAZ*-complemented strains by the infiltration method at 2×10^8 colony-forming units (cfu)/mL. The induction of *HIN1* expression was prominent after inoculation with the *hrpAZ*- and *hrpZ*-complemented strains, although *HIN1* was not remarkably induced by inoculation with the *Pta* 6605 WT strain (Fig. 3B).

The recombinant 42-kDa harpin$_{Pss}$ (harpin from *Pss*) is known to effectively induce defence responses in tobacco (>2.4 μM), potato (>2.4 μM) and tomato (>20 μM), but not in bean at a concentration of 60 μM (He *et al*., 1993). Similarly, harpin$_{Pss}$, harpin$_{Pgl}$ and harpin$_{Pto}$ (harpins from *Pss*, *Pgl* and *Pto*, respectively) elicit HR in tomato but not in soybean (Preston *et al*., 1995). We also observed that the recombinant harpin$_{Pto}$, harpin$_{Pgl}$ and harpin$_{Ppi}$ (harpin from *Ppi*) did not elicit pea defence responses at a concentration of 6.8 μM (Tanaka *et al*., 2001). Thus, the effects of harpin vary among plant species, and leguminous plants seem to be insensitive or slightly sensitive to harpins from *P. syringae*. The reason for the differential response in plants to harpin protein may depend on the existence of corresponding receptors in plants and quantitative differences in behaviour in harpin recognition. He *et al*. (1993) also found that a recombinant 32-kDa harpin protein (harpin$_{Pss}$Δ125), in which 125 N-terminal amino acids were deleted, induced a rather higher defence response. For example, harpin$_{Pss}$Δ125 induced a defence response in tobacco (>0.6 μM), tomato (>5 μM) and potato (>0.6 μM), indicating that the 125 N-terminal amino acids in harpin$_{Pss}$ are not required to induce the plant defence response (He *et al*., 1993). To define the smallest fragment responsible for elicitor activity, a variety of sizes and domains of recombinant harpin$_{Pph}$ polypeptides (harpins from *Pph*) were expressed in *E. coli*, and their elicitor activity was assessed in tobacco (Lee *et al*., 2001a). The results suggest that the elicitor activity of recombinant harpin$_{Pph}$ resides in a carboxy (C)-terminal fragment corresponding to amino acids 200–300 (Lee *et al*., 2001a). Furthermore, harpin$_{Pss}$ lacking an amino acid sequence (254–298) and harpin$_{Pph}$ lacking a C-terminal sequence (306–345) lost HR-inducing activity (Haapalainen *et al*., 2010;

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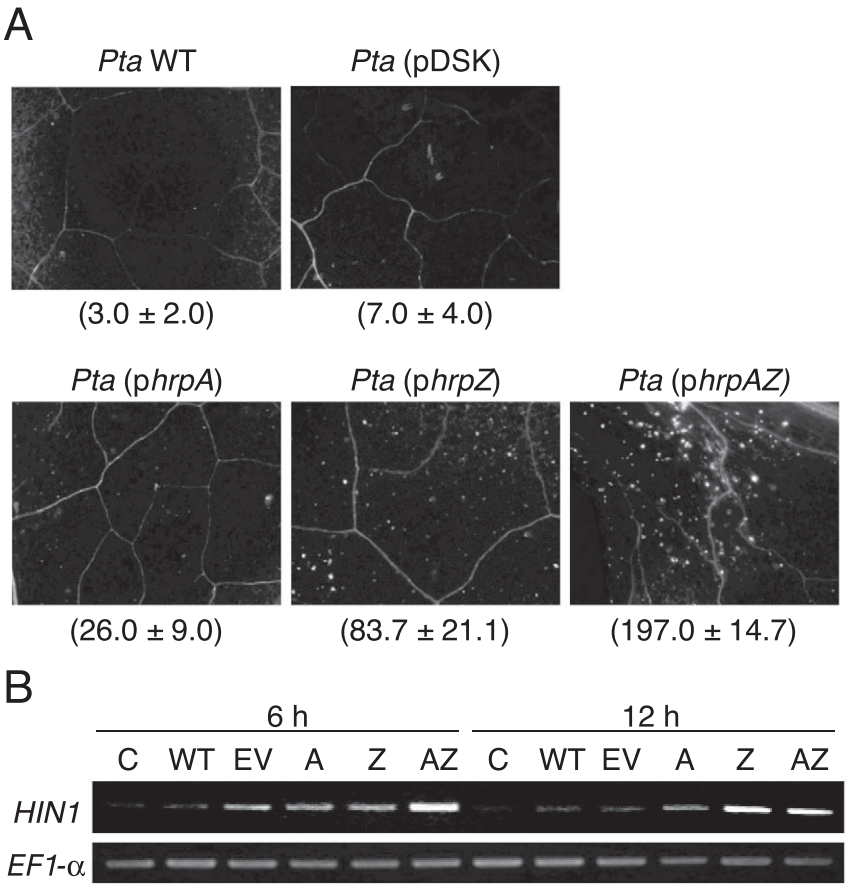

Fig. 3 Tobacco defence responses. Tobacco leaves were infiltration inoculated with *Pseudomonas syringae* pv. *tabaci* (*Pta*) 6605 [*Pta* wild-type (WT)] or *Pta* (p*hrpAZ*). (A) Tobacco leaves were incubated for 48 h, and then stained with aniline blue according to the method described by Adam and Somerville (1996) to observe callose deposition. The average number (±standard deviation) of callose deposits per 4-mm^2 area is shown in parentheses. (B) Gene expression of *HIN1* (AF212183) was investigated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). One microgram of total RNA was used to synthesize the first-strand cDNA with AMV RTase according to the manufacturer's protocols (Takara, Otsu, Japan). The PCR programme involved one cycle of 95°C for 2 min, followed by 28 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 1 min. The expression of *elongation factor 1*α (*EF-1*α, D63396) was determined as a housekeeping gene. Primer sequences used for PCR were as follows: *NtHIN1* (5′-caaggcgagaggtttgatag-3′, 5′-gatggcatctggtttcctca-3′) and *NtEF-1*α (5′-tcacatcaacattgtggtcattggc-3′, 5′-tggtagcatccatcttgttacagca-3′). Experiments were performed in triplicate and representative results are shown.

He *et al*., 1993). Haapalainen *et al*. (2010) also reported that tobacco recognizes a P24 peptide (amino acids 290–313, PNQDLGQLLGGLLQKGLEATLQDA), which resides in the C-terminal domain from *Pph* HrpZ and elicits a full HR in tobacco. Because the truncated *Pta* harpin protein also lacks the C-terminal domain, including P24 (Fig. S1), our observation is consistent with the results of He *et al*. (1993), Lee *et al*. (2001a) and Haapalainen *et al*. (2010). Although the structurally minimal portion of harpin that elicits plant defence was not clearly identified in other pathovars, a deleted portion of harpin$_{Pta}$ might contain the elicitor-active domain in part.

Previously, Huang *et al*. (1988) have reported that the cosmid clone pHIR11 containing a 31-kb DNA from *Pss* 61 is responsible for the elicitation of plant reactions. A pHIR11 cosmid that contains the entire *hrp* gene cluster and *hrp*-linked *hrmA* has been reported to contain two regions that alkalinize the medium of tobacco suspension cell cultures (Huang *et al*., 1988, 1995). *HrmA* was found to encode an Avr-like protein, HopPsyA, that converts compatible tobacco–pathogen interactions into incompatible interactions (Alfano *et al*., 1997; Shen *et al*., 2000). However, the *hrmA* homologue was absent in *Pta* 11528, and thus heterologous expression of *hrmA* from pHIR11 enhanced the tobacco defence response (Alfano *et al*., 1997). Very recently, it has been revealed that there is also no *hrmA* homologue in *Pta* 6605 (D. Studholme, University of Exeter, personal communication). In this study, we found that harpin functions as an avirulence factor when expressed in *Pta*, although harpin is not strictly an avirulence factor because it is not race–cultivar specific, but rather pathovar–plant species' specific, and the corresponding resistance gene for the *hrpZ* gene is not known.

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Although *hrpZ*- and *hrpAZ*-complemented strains did not cause disease symptoms, they were able to survive and multiply to some extent in tobacco leaves, indicating that the disruption of *hrpZ* is not the only adaptation of *Pta* that allows this bacterial strain to live in tobacco plants. It is well known that *Pta* secretes a variety of virulence factors, such as effectors and tabtoxin, that allow its growth in tobacco leaves. These factors might suppress strong harpin-induced defence responses, including HR. We observed that the inoculation of tobacco leaves with *Pta* (p*hrpZ*) and *Pta* (p*hrpAZ*) did not cause HR, and that all bacteria isolated from tobacco leaves 6 days after inoculation with *Pta* (p*hrpAZ*) were kanamycin resistant. These results indicate that the plasmid was not cured from bacteria during the infection process, and it is possible that the *hrpZ*-complemented strains may not secrete sufficient harpin to induce HR cell death.

Comparison of the phenotypes of *Pta* (p*hrpAZ*) and *Pta* (p*hrpZ*) shows that the reduction in virulence is more prominent in *Pta* (p*hrpAZ*) (Fig. 2A), and that *Pta* (p*hrpAZ*) induces callose deposition and *HIN1* gene expression more strongly than *Pta* (p*hrpZ*) (Fig. 3). It is not known whether or not HrpA has elicitor activity. However, if HrpA has weak elicitor activity, these results can be explained as tobacco defence responses induced by the expression of an elicitor protein HrpA.

In the phylogenetic analyses of *P. syringae* pathovars, DNA sequences for *hrp* and its neighbouring regions were investigated, and *Pta* was classified in the same group with *Pgl* and *Pph* (Guttman *et al*., 2006; Inoue and Takikawa, 2000; Sawada *et al*., 1999). Guttman *et al*. (2006) carried out the comparative sequencing analysis of 22 *hrpZ* operons from *P. syringae* strains, and reported that *hrpA* is under diversifying selection to maintain genetic diversity in order to avoid detection by the host's innate immune system, whereas *hrpZ* is not under strong selective pressure. However, Guttman *et al*. (2006) did not include *hrpZ* of *Pta* in the construction of phylogenetic trees for their analysis. Thus, Guttman's result and our study indicate that, except for *Pta*, *hrpZ* is relatively stable among *P. syringae* pathovars, and that the internal deletion in *hrpZ* is specific to *Pta*.

Although the intrinsic function of harpin protein in pathogenesis is not fully understood, based on the evidence that harpin$_{Pph}$ binds to lipid bilayers and forms an ion-conducting pore (Lee *et al*., 2001b), it is thought that harpin allows nutrient release and/or delivery of virulence factors during bacterial colonization through the host plasma membrane. The HrpN$_{Ea}$ harpin of *E. amylovora* triggered defence responses on its nonhost *Arabidopsis thaliana* cells but not on host apple cells (Reboutier *et al*., 2007). Reboutier *et al*. (2007) hypothesized that HrpN$_{Ea}$ harpin assists the translocation of effector proteins into plant cells. Some nonhost plants seem to be able to recognize HrpN$_{Ea}$ harpin and trigger defence responses; however, apple cells are probably missing a guard receptor able to recognize harpin and induce defence responses. Furthermore, Bocsanczy *et al*. (2008) demonstrated that HrpN$_{Ea}$ harpin was involved in the delivery of DspA/E, an indispensable virulence factor of *E. amylovora*. Recently, Engelhardt *et al*. (2009) reported that the intrinsic function of HrpZ1 harpin from *Pph* is to permeate membranes during host infection, and that the pore formation and plant immunity-stimulating activities of HrpZ1 harpin are structurally separable. Namely, a C-terminal fragment of HrpZ1 harpin retains the ability to activate plant immunity, whereas ion pore formation requires intact HrpZ1.

We speculate that, when the ancestor of *Pta* attempted to invade tobacco plants, harpin might have been one of the virulence factors. However, after tobacco plants acquired a receptor molecule for harpin protein and established the mechanisms to trigger strong and rapid harpin-mediated defence responses, the ancestor of *Pta* apparently lost its virulence on tobacco plants. Thus, *Pta* might have adapted to the tobacco plant by disruption of the *hrpZ* gene to evade recognition, and might have again acquired virulence on tobacco plants. Such co-evolution of host plants to overcome pathogenic invasion and of phytopathogens to evade host detection and/or to suppress the expression of defence response is often called an arms race (Ingle *et al*., 2006). Thus, the deletion of *hrpZ* in *Pta* is a typical example of an evolutionary arms race between plants and phytopathogenic bacteria.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Deduced amino acid sequences of harpin proteins from *Pseudomonas syringae* pv. *tabaci* (*Pta*) (Taguchi *et al*., 2001), *P. syringae* pv. *phaseolicola* (*Pph*) (Lee *et al*., 2001a) and *P. syringae* pv. *syringae* (*Pss*) (He *et al*., 1993). The amino acid sequence from *Pta* is frameshifted by the deletion at position 131, and the nonhomologous extension is represented by lowercase letters. The peptide sequence represented by red letters (P24) in *Pph* elicits a full hypersensitive response (HR) in tobacco (Haapalainen *et al*., 2010). Peptide sequences underlined are reported to be necessary for HR induction in *Pph* (Haapalainen *et al*., 2010) and *Pss* (He *et al*., 1993).

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