

# Catalytic domain of the diversified *Pseudomonas syringae* type III effector HopZ1 determines the allelic specificity in plant hosts

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## Summary

The type III secretion systems (T3SS) and secreted effectors (T3SEs) are essential virulence factors in Gram-negative bacteria. During the arms race, plants have evolved resistance (R) genes to detect specific T3SEs and activate defence responses. However, this immunity can be efficiently defeated by the pathogens through effector evolution. HopZ1 of the plant pathogen *Pseudomonas syringae* is a member of the widely distributed YopJ T3SE family. Three alleles are known to be present in *P. syringae*, with HopZ1a most resembling the ancestral allelic form. In this study, molecular mechanisms underlying the sequence diversification-enabled HopZ1 allelic specificity is investigated. Using domain shuffling experiments, we present evidence showing that a central domain upstream of the conserved catalytic cysteine residue determines HopZ1 recognition specificity. Random and targeted mutagenesis identified three amino acids involved in HopZ1 allelic specificity. Particularly, the exchange of cysteine141 in HopZ1a with lysine137 at the corresponding position in HopZ1b abolished HopZ1a recognition in soybean. This position is under strong positive selection, suggesting that the cysteine/lysine mutation might be a key step driving the evolution of HopZ1. Our data support a

model in which sequence diversification imposed by the plant R gene-associated immunity has driven HopZ1 evolution by allowing allele-specific substrate-binding.

## Introduction

Pathogens and their hosts are engaged in an endless and deadly warfare. These interactions are mediated by the diverse suites of virulence factors from the pathogens and the surveillance systems from the hosts (Ma and Guttman, 2008). The dynamic nature of pathogen–host interaction is best described in a zigzag model with a stepwise pattern representing different stages of the interactions (Jones and Dangl, 2006).

Host–pathogen interactions are generally believed to start with the host recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Abramovitch *et al.*, 2006a; Chisholm *et al.*, 2006). The perception of PAMPs or MAMPs triggers a basal defence response called *PAMP-triggered immunity* (PTI), which effectively restricts the growth of the vast majority of potential pathogens in the hosts. Successful pathogens produce a variety of virulence factors to suppress PTI. In Gram-negative bacteria, PTI can be suppressed through the action of effector proteins secreted by the type III secretion system (T3SS). The T3SS is a specialized protein delivery system that injects virulence proteins directly into the host cytosol in order to facilitate bacterial infection (Alfano and Collmer, 1997; He *et al.*, 2004; Galan and Wolf-Watz, 2006). Several type III secreted effectors (T3SEs) have been shown to efficiently block host disease resistance mechanisms, including PTI, and swing the outcome of the interaction back to the disease state (Grant *et al.*, 2006; He *et al.*, 2006; Rosebrock *et al.*, 2007; Guo *et al.*, 2009). For these specialized pathogens, plants have evolved a second layer of defence based on resistance (R) proteins, which recognize specific T3SEs and restrict pathogen growth by triggering a localized and rapid programmed cell death referred to as the hypersensitive response (HR) (Greenberg, 1997; Heath, 2000).

R protein-based effector-triggered immunity (ETI) imposes intense selective pressures on the pathogens. In

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response to these pressures, three major evolutionary routes could be taken by the pathogens to shift the balance of power from plants to pathogens: (i) inactivation of the effector protein to evade the recognition (Stevens *et al.*, 1998; Gassmann *et al.*, 2000; Pitman *et al.*, 2005; Wichmann *et al.*, 2005; Ma *et al.*, 2006); (ii) acquisition of T3SEs that act at or downstream of R protein recognition to block the host signal transduction pathways leading to ETI (Jamir *et al.*, 2004; Abramovitch *et al.*, 2006b; Rosebrock *et al.*, 2007); (iii) replacement of the effector with a functionally equivalent allele through either mutational changes or horizontal gene transfer. The third route is a specifically important strategy for pathogens to defeat ETI and regain virulence in cases when inactivation of the effector would cause a significant reduction on pathogen fitness *in planta*. Sequence diversification-enabled effector evolution has been found in bacteria (Keith *et al.*, 1997; Stevens *et al.*, 1998; Rohmer *et al.*, 2004), oomycetes (Allen *et al.*, 2004; Win *et al.*, 2007) and fungal pathogens (Dodds *et al.*, 2006; Stergiopoulos *et al.*, 2007). However, the molecular basis driving and maintaining the evolution of these diversified effectors is still lacking, especially in bacterial pathogens.

In this study, the evolutionarily conserved and diverse HopZ T3SE family in the plant pathogen *Pseudomonas syringae* is used as a model to investigate the molecular basis underlying sequence diversification-enabled effector evolution. HopZ homologues belong to the YopJ family of T3SEs, which have been identified from both plant and animal pathogens (Arnold *et al.*, 2001; Guttman *et al.*, 2002; Orth, 2002; Charity *et al.*, 2003; Deng *et al.*, 2003; Sundin *et al.*, 2004). Members of the YopJ family T3SEs all share a conserved catalytic core, consisting of three key amino acid residues (histidine, glutamic acid and cysteine), that is identical to that of the C55 family of cysteine proteases (Barrett and Rawlings, 2001). However, YopJ has been shown to possess an acetyltransferase activity and suppress host immune response in animals by blocking the activation of mitogen-activated protein kinases through acetylating the serine and threonine residues in their activation loops (Mukherjee *et al.*, 2006). Although the enzymatic activity of HopZ homologues *in planta* remains to be determined, the predicted catalytic cysteine residues are required for their cellular functions (Lewis *et al.*, 2008; Zhou *et al.*, 2009).

The HopZ homologues comprise three major homology groups (named HopZ1, Z2 and Z3) that are widely distributed in *P. syringae* strains (Ma *et al.*, 2006). Evolutionary and phylogenetic analyses suggested that HopZ1 was an ancestral virulence factor in *P. syringae* that was under strong diversifying and positive selections. At least three functional allelic classes (HopZ1a, HopZ1b and HopZ1c) and two degenerate alleles of HopZ1 have evolved through mutational changes with HopZ1a most resem-

bling the allele carried by the ancestral *P. syringae* strain (Ma *et al.*, 2006). Introduction of exogenous HopZ1a into *P. syringae* strains harbouring alternative or degenerate HopZ1 alleles results in HR in their respective hosts, which is not observed with the endogenous allele (Ma *et al.*, 2006). For example, soybean is a natural host of *P. syringae* pv. *glycinea* (*Pgy*) strains, which all produce the HopZ1b allele. However, *Pgy* strains expressing HopZ1a trigger HR in soybean (Ma *et al.*, 2006).

HopZ1b, derived from a HopZ1a-like ancestor, shares approximately 81% similarity in the full-length amino acid sequence with HopZ1a. Although a HopZ1b-deleted mutant of *Pgy* strain BR1 (*Pgy*BR1) did not show an observable growth defect on soybean cultivar OAC Bayfield, a significant growth advantage is conferred by HopZ1b to the growth of a non-pathogenic *P. syringae* strain on soybean (Zhou *et al.*, 2009). Functional redundancy is very common for T3SEs in *P. syringae* (Alfano and Collmer, 2004; Kvitko *et al.*, 2009). It is likely that the virulence function of HopZ1b on soybean is redundant with other T3SE(s) in *Pgy*BR1 under our experimental conditions. Nevertheless, the ability to promote non-host bacterial growth and the ubiquitous distribution in *Pgy* strains strongly suggests that HopZ1b plays a role in facilitating bacterial infection on soybean.

These findings suggest that HopZ1 and its corresponding plant R proteins are engaged in a co-evolutionary arms race. It is likely that the recognition of the ancestral HopZ1a-like allele by plant R protein-associated defence system has selected for mutated alleles no longer recognized by the host while retaining the virulence functions. In the case of HopZ1b, effector evolution was accomplished through sequence diversification.

Here, we investigated the molecular basis of the sequence diversification-enabled allelic specificity of HopZ1. Domain shuffling and mutagenesis experiments were employed to identify key sequences and amino acid residues responsible for the distinct cellular functions of HopZ1 alleles in plant hosts. We present evidence to demonstrate that the central domains of HopZ1 (HopZ1a<sub>63-203</sub> and the corresponding region HopZ1b<sub>59-199</sub>) upstream of the predicted catalytic cysteine residue (C216 in HopZ1a and C212 in HopZ1b) determine the allelic specificity. Furthermore, the mutation in the amino acid 141 from a cysteine in HopZ1a to a lysine, as in the corresponding position in HopZ1b, abolishes the HR-triggering activity of HopZ1a in soybean. This residue is under strong positive selection, suggesting that it may be involved in HopZ1 allelic specificity. We hypothesize that the cysteine to lysine mutation may significantly contribute to the alternation of the substrate-binding specificity of HopZ1b, which leads to recognition evasion and maintenance of virulence function of this newly evolved allele in soybean. Therefore, this mutational change may

be a key step during HopZ1 evolution to evade host recognition.

## Results and discussions

### *A central domain upstream of the catalytic cysteine determines HopZ1 allelic specificity*

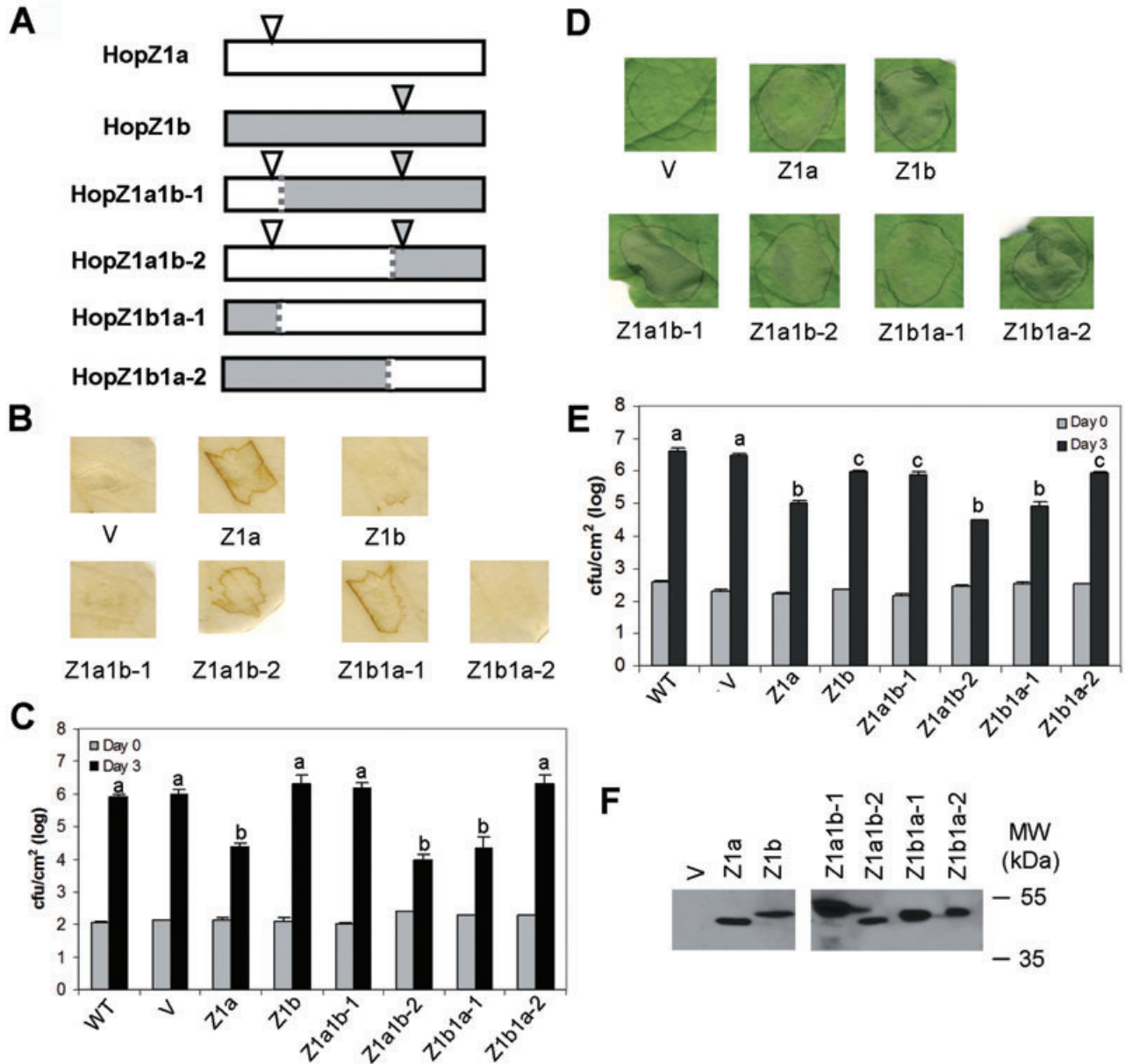
HopZ1a and HopZ1b share 72% identity and 81% similarity in their full-length amino acid sequences with two major indels, 28–31 aa of HopZ1a and 251–253 aa of HopZ1b (represented by the white and grey triangles, respectively, in Fig. 1A). The regions surrounding these indels also have relatively high sequence variability between the two alleles, compared with the rest of the protein. To determine whether these two variable regions were responsible for the distinct plant responses induced by HopZ1a and HopZ1b, we constructed four chimeric proteins by fusing the N-terminus of HopZ1a or HopZ1b with the C-terminus of HopZ1b or HopZ1a respectively (Fig. 1A). These chimeric proteins are designated HopZ1a1b-1 (HopZ1a<sub>1–62</sub> fused to HopZ1b<sub>59–368</sub>), HopZ1a1b-2 (HopZ1a<sub>1–244</sub> fused to HopZ1b<sub>240–368</sub>), HopZ1b1a-1 (HopZ1b<sub>1–58</sub> fused to HopZ1a<sub>63–369</sub>) and HopZ1b1a-2 (HopZ1b<sub>1–239</sub> fused to HopZ1a<sub>245–369</sub>). The chimeric genes were ligated into the plasmid vector pUCP20tk (Zhou *et al.*, 2009) with their expression under the control of the native promoter of either hopZ1a or hopZ1b, depending on which allele was at the N-terminus of a particular chimeric protein.

pUCP20tk carrying the wild-type and chimeric *hopZ1* genes were introduced into *P. syringae* pv. *glycinea* strain BR1Rif-O1 (*PgyBR1Rif-O1*). *PgyBR1Rif-O1* is a mutant of the wild-type bacterium *PgyBR1Rif* with the endogenous plasmid carrying the *hopZ1b* gene cured (Zhou *et al.*, 2009). Therefore, this strain provides a clean genetic background for expressing different *hopZ1* alleles in order to study their phenotypes during interaction with plant hosts. *PgyBR1Rif-O1* expressing the wild-type and chimeric HopZ1 proteins was infiltrated into the unifoliates of nine-day old soybean seedlings (*Glycine max* cv. Williams), and their HR-eliciting activities were examined. Similar to HopZ1a, HopZ1a1b-2 and HopZ1b1a-1, both containing the central domain of HopZ1a, triggered HR in soybean (Fig. 1B). On the contrary, HopZ1a1b-1 and HopZ1b1a-2, both containing the central domain of HopZ1b, did not trigger HR in soybean, similar to HopZ1b (Fig. 1B). The HR-eliciting activities of the chimeric proteins were confirmed using the *in planta* bacterial multiplication assays, which demonstrated that the bacterial populations of *PgyBR1Rif-O1* expressing HopZ1a1b-2 and HopZ1b1a-1 were similar to that of *PgyBR1Rif-O1* expressing the wild-type HopZ1a and were significantly lower than that of *PgyBR1Rif-O1* expressing HopZ1a1b-1 or HopZ1b1a-2 (Fig. 1C). The normal expression of these

chimeric proteins in *PgyBR1Rif-O1* induced in M63 minimal medium was verified using Western blots, confirming that the defective HR elicitation of HopZ1a1b-1 and HopZ1b1a-2 was not due to the lack of protein expression (Fig. 1F).

Genes encoding these HopZ1 chimeric proteins were also cloned in the plasmid vector pMDD1 (Mudgett *et al.*, 2000) and transiently expressed in *N. benthamiana* through *Agrobacterium*-mediated infiltration to examine their cell death-eliciting activities. We have previously shown that HopZ1a and HopZ1b both trigger HR in *N. benthamiana*. However, they appear to induce defence responses through different pathways. Visually, HopZ1a and HopZ1b trigger different cell death symptoms when transiently expressed in *N. benthamiana* (Zhou *et al.*, 2009). *Agrobacterium*-mediated expression of HopZ1a1b-1 and HopZ1b1a-2 resulted in a relatively stronger cell death symptom that started to appear at approximately 36 h post inoculation (hpi), similar to that of HopZ1b (Fig. S1). However, HopZ1a1b-2 and HopZ1b1a-1, containing the central domain of HopZ1a, induced a weaker cell death symptom that was visible after approximately 60 hpi, similar to that of HopZ1a (Fig. S1).

In order to develop a quantitative evaluation of HopZ1 allele-specific HR elicitation in *N. benthamiana*, we infiltrated the leaves of 4-week-old *N. benthamiana* plants with *PgyBR1Rif-O1* expressing HopZ1a and HopZ1b and measured the bacterial populations 3 days post inoculation. Consistently with the *Agrobacterium*-mediated transient expression, both HopZ1a and HopZ1b triggered HR (Fig. 1D) and the bacterial populations of *PgyBR1Rif-O1* expressing HopZ1a and HopZ1b were largely decreased compared with that of *PgyBR1Rif-O1* carrying the empty vector (Fig. 1E). However, *PgyBR1Rif-O1* expressing HopZ1b multiplied to a higher level than *PgyBR1Rif-O1* expressing HopZ1a in *N. benthamiana* (Fig. 1E). These results seem to be the opposite from the transient expression assay, where HopZ1b triggers a faster and stronger HR than HopZ1a in *N. benthamiana*. It is possible that there are other factors, such as some T3SEs in *PgyBR1Rif-O1* that can partially suppress the HopZ1b-triggered HR. For example, we have previously shown that HopZ3 inhibits HopZ1b-, but not HopZ1a-triggered HR in *N. benthamiana* (Zhou *et al.*, 2009). Nevertheless, the *in planta* bacterial multiplication assay provided a sensitive method to evaluate the allelic specificity of HopZ1a and HopZ1b in *N. benthamiana*. Consistently with the soybean assays, *PgyBR1Rif-O1* expressing HopZ1a1b-1 and HopZ1b1a-2 multiplied to a similar level as *PgyBR1Rif-O1* expressing the wild-type HopZ1b; and *PgyBR1Rif-O1* expressing HopZ1a1b-2 and HopZ1b1a-1 multiplied to a similar level as *PgyBR1Rif-O1* expressing the wild-type HopZ1a (Fig. 1E).



Experiments on soybean and *N. benthamiana* clearly demonstrated that neither of the two major variable regions in HopZ1 was involved in allelic specificity. Rather, the central domain (63–244 aa of HopZ1a, corresponding to 59–240 aa of HopZ1b) containing the predicted conserved catalytic core determined the allele-specific recognition of HopZ1 in soybean and *N. benthamiana*.

Members of the YopJ family T3SEs all share conserved catalytic cores consisting of three key amino acid residues (histidine, glutamic acid and cysteine), which are identical to that of the C55 family of cysteine proteases (Barrett and Rawlings, 2001). In HopZ1a, the catalytic triad is H151, E170 and C216; and in HopZ1b, the three catalytic resi-

dues are H147, E166 and C212. Although YopJ has been demonstrated to be an acetyltransferase, the same catalytic triad is required for the acetyltransferase activity (Mittal *et al.*, 2006; Mukherjee *et al.*, 2006). Thus far, the specific enzymatic activity of HopZ proteins *in planta* has not been described. Nevertheless, the predicted catalytic cysteine residues are essential for their cellular functions in plants (Ma *et al.*, 2006; Lewis *et al.*, 2008; Zhou *et al.*, 2009), indicating that the enzymatic activity is required for HopZ effectors to perform their biological functions. Together with these prior findings, the domain shuffling data showing that the allelic specificity of HopZ1a and HopZ1b is determined by the central domain containing the catalytic core indicate that the HopZ1 allelic specificity

**Fig. 1.** Effect of HopZ1 chimeric proteins on the bacterial growth of *Pseudomonas syringae* pv. *glycinea* strain BR1Rif-O1 (*PgyBR1Rif-O1*) in soybean and *N. benthamiana*. All the experiments were repeated three times with similar results and data from one experiment are shown.

A. Construction of HopZ1 chimeric proteins by domain shuffling. The triangles represent the two major indels: 28–31 aa of HopZ1a is indicated by the open triangle and 251–253 aa of HopZ1b is indicated by the grey triangle. The dotted lines represent the junctions of the HopZ1a-1b chimeric proteins. HopZ1a1b-1 has HopZ1a<sub>1–62</sub> fused to HopZ1b<sub>59–368</sub>; HopZ1a1b-2 has HopZ1a<sub>1–244</sub> fused to HopZ1b<sub>240–368</sub>; HopZ1b1a-1 has HopZ1b<sub>1–58</sub> fused to HopZ1a<sub>64–369</sub>; HopZ1b1a-2 has HopZ1b<sub>1–238</sub> fused to HopZ1a<sub>245–369</sub>.

B. Soybean HR assays of *PgyBR1Rif-O1* expressing the wild-type and chimeric genes carried on the plasmid vector pUCP20tk. Unifoliates of 14-day-old soybean plants were pressure infiltrated with *PgyBR1Rif-O1* expressing the wild-type and chimeric proteins (OD<sub>600</sub> = 0.3). The empty pUCP20tk vector (V) was used as a control. Soybean leaves were harvested 48 h post inoculation and photographed after ethanol bleaching.

C. Soybean *in planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing the wild-type and chimeric HopZ1 proteins. The empty pUCP20tk vector (V) was used as a control. Bacterial cell suspensions (OD<sub>600</sub> = 0.0001) were infiltrated into the unifoliates of 9-day-old soybeans. Colony-forming units were determined 0 and 3 dpi (days post inoculation). Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

D. HR assay of *PgyBR1Rif-O1* expressing the wild-type and chimeric HopZ1 proteins on *N. benthamiana*. Four-week-old *N. benthamiana* plants were pressure infiltrated with *PgyBR1Rif-O1* expressing HopZ1a, HopZ1b, HopZ1a1b-1, HopZ1a1b-2, HopZ1b1a-1 and HopZ1b1a-2 carried on the plasmid vector pUCP20tk (OD<sub>600</sub> = 0.01). The empty pUCP20tk vector (V) was used as a control. *N. benthamiana* leaves were harvested and photographed 48–72 h post inoculation.

E. *In planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing the wild-type and chimeric HopZ1 proteins in *N. benthamiana*. Bacterial cell suspensions (OD<sub>600</sub> = 0.0001) were infiltrated into the leaves of 4-week-old *N. benthamiana*. The empty pUCP20tk vector (V) was used as a control. Colony-forming units were determined 0 and 3 dpi. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

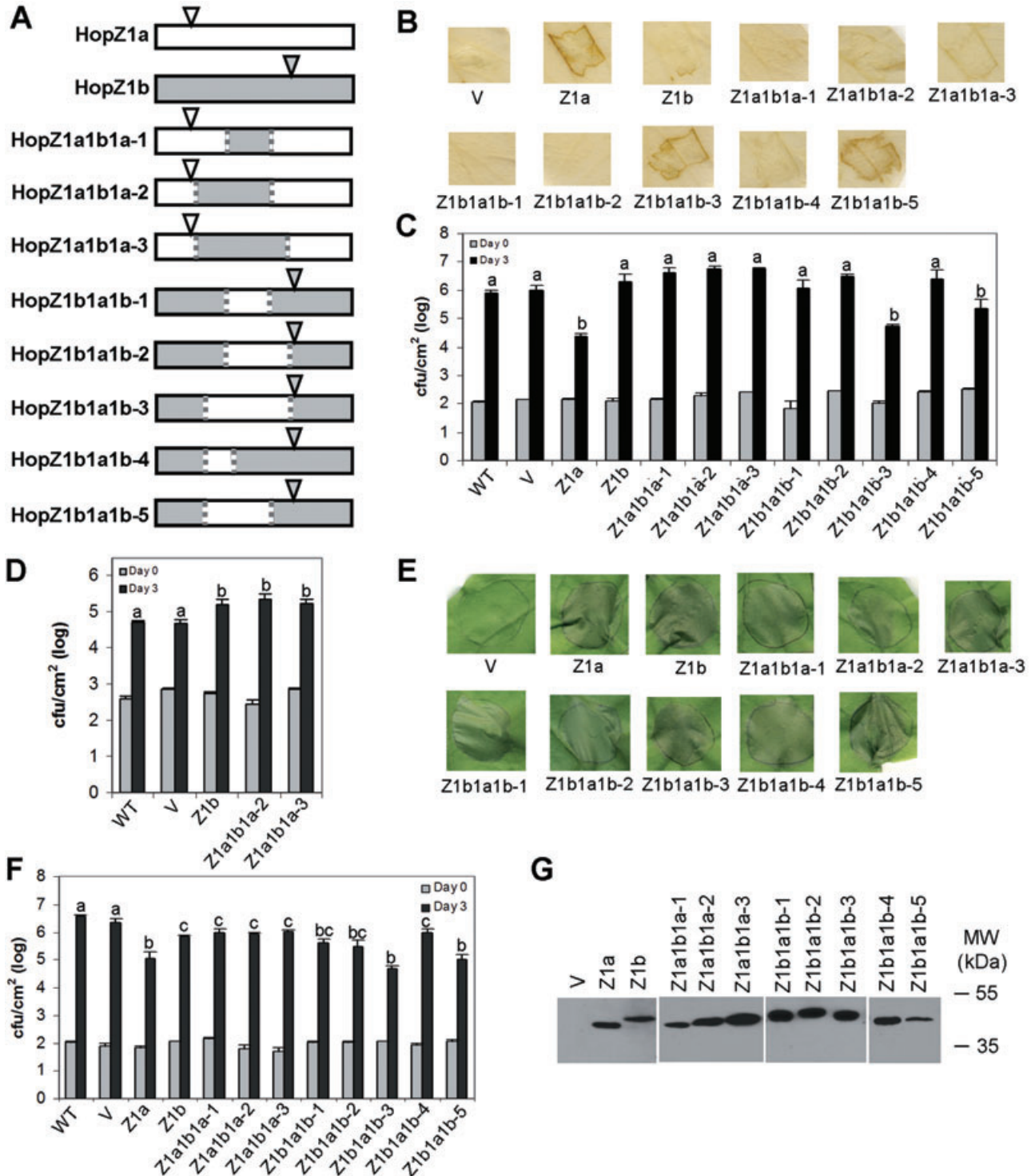
F. Immunoblot showing the wild-type and chimeric HopZ1 protein expression in *PgyBR1Rif-O1*. *PgyBR1Rif-O1* cells expressing HopZ1 proteins were induced in M63 minimal medium containing 1% fructose overnight at room temperature. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by anti-HA antibody conjugated with HRP.

is probably resulted from allelic specific enzymatic characterizations.

In order to further define and verify the key sequence for the allelic specificity within the central region of HopZ1, eight additional ‘double-chimeric’ proteins were constructed (Fig. 2A). Three ‘double-chimeric’ proteins contain the N-terminal and C-terminal sequences of HopZ1a with the corresponding HopZ1b sequence in the middle: HopZ1a1b1a-1 contains HopZ1a<sub>1–137</sub> fused to HopZ1b<sub>134–199</sub> and then HopZ1a<sub>204–369</sub>; HopZ1a1b1a-2 contains HopZ1a<sub>1–62</sub> fused to HopZ1b<sub>59–199</sub> and then HopZ1a<sub>204–369</sub>; HopZ1a1b1a-3 contains HopZ1a<sub>1–62</sub> fused to HopZ1b<sub>59–240</sub> and then HopZ1a<sub>245–369</sub>. The other five ‘double-chimeric’ proteins contain the N-terminal and C-terminal sequences of HopZ1b with the corresponding HopZ1a sequence in the middle: HopZ1b1a1b-1 contains HopZ1b<sub>1–133</sub> fused to HopZ1a<sub>138–203</sub> and then HopZ1b<sub>200–368</sub>; HopZ1b1a1b-2 contains HopZ1b<sub>1–133</sub> fused to HopZ1a<sub>138–244</sub> and then HopZ1b<sub>241–368</sub>; HopZ1b1a1b-3 contains HopZ1b<sub>1–58</sub> fused to HopZ1a<sub>63–244</sub> and then HopZ1b<sub>241–368</sub>; HopZ1b1a1b-4 contains HopZ1b<sub>1–58</sub> fused to HopZ1a<sub>63–137</sub> and then HopZ1b<sub>134–368</sub>; HopZ1b1a1b-5 contains HopZ1b<sub>1–58</sub> fused to HopZ1a<sub>63–203</sub> and then HopZ1b<sub>200–368</sub>. These domain shuffling products were cloned into the vector pUCP20tk and expressed in *PgyBR1Rif-O1*. The expression of the HopZ1a1b1a constructs are under the control of the *hopZ1a* promoter, and the expression of HopZ1b1a1b constructs are under the control of the *hopZ1b* promoter. All the chimeric proteins are expressed at a similar level in *PgyBR1Rif-O1* as the wild-type HopZ1 proteins when the cells were induced in the M63 minimal medium (Fig. 2G).

The ‘double-chimeric’ proteins were examined for the HR-eliciting activities in soybean by infiltration assays (Fig. 2B) and *in planta* bacterial multiplication assays (Fig. 2C). None of the HopZ1a1b1a constructs triggered HR in soybean. HopZ1a1b1a-1, with the region 138–203 aa in HopZ1a replaced by the corresponding sequence of HopZ1b, contains the shortest HopZ1b sequence, suggesting that this region is required for HopZ1a to trigger HR. Furthermore, the mutant HopZ1b1a1b-1, carrying the region 138–203 aa of HopZ1a in HopZ1b, did not gain HR-triggering activity, suggesting that the region HopZ1a<sub>138–203</sub> was not sufficient to induce HR in soybean. In fact, only *PgyBR1Rif-O1* expressing HopZ1b1a1b-3 (carrying HopZ1a<sub>63–244</sub>) and HopZ1b1a1b-5 (carrying HopZ1a<sub>63–203</sub>) elicited HR, suggesting that the substitution of HopZ1b<sub>59–199</sub> with the corresponding HopZ1a<sub>63–203</sub> was sufficient to trigger HR in soybean. Since neither the HopZ1b1a1b-4 mutant containing the region HopZ1a<sub>63–137</sub>, nor the HopZ1b1a1b-1 mutant containing the region HopZ1a<sub>138–203</sub> was sufficient for HR-elicitation, these observations suggested that both HopZ1a<sub>138–203</sub> and its immediate upstream region HopZ1a<sub>63–137</sub> are required for the HR-eliciting activity of HopZ1a in soybean.

HopZ1b was previously shown to promote the multiplication of a non-host pathogen, *P. syringae* pv. *phaseolicola* (*Pph*) strain 1302ARif (*Pph1302ARif*) in soybean (Zhou *et al.*, 2009). According to the HR assay data showing that replacement of HopZ1b<sub>59–199</sub> with the corresponding HopZ1a sequence (HopZ1a<sub>63–203</sub>) is sufficient to trigger HR in soybean, we then examined the constructs HopZ1a1b1a-2 (containing HopZ1b<sub>59–199</sub>) and HopZ1a1b1a-3 (containing HopZ1b<sub>59–240</sub>) for their ability



to promote *Pph1302ARif* multiplication in soybean. *Pph1302ARif* expressing HopZ1a1b1a-2 and HopZ1a1b1a-3, carried on the vector pUCP20tk, were infiltrated into soybean and the bacterial populations were evaluated 3 days post inoculation (Fig. 2D). Similar to HopZ1b, HopZ1a1b1a-2 and HopZ1a1b1a-3 both pro-

moted the *in planta* multiplication of *Pph1302ARif*, indicating that HopZ1b<sub>59-199</sub> is sufficient to confer the non-host bacterial growth promotion in soybean.

All the 'double-chimeric' proteins were also examined for the allele-specific HR-eliciting activities in *N. benthamiana* using *PgyBR1Rif*-O1-mediated HR assays (Fig. 2E)

**Fig. 2.** Effect of HopZ1 double-chimeric proteins on the bacterial growth of *Pseudomonas syringae* pv. *glycinea* strain BR1Rif-O1 (*PgyBR1Rif-O1*) in soybean and *N. benthamiana*. All the experiments were repeated at least three times with similar results and data from one experiment are shown.

A. Construction of HopZ1 double-chimeric proteins by domain shuffling. The triangles and the dotted lines represent the two indels and the junctions of the chimeric proteins respectively. HopZ1a1b1a-1 has HopZ1a<sub>1-137</sub> fused to HopZ1b<sub>134-199</sub> and then HopZ1a<sub>204-369</sub>; HopZ1a1b1a-2 has HopZ1a<sub>1-62</sub> fused to HopZ1b<sub>59-199</sub> and then HopZ1a<sub>204-369</sub>; HopZ1a1b1a-3 has HopZ1a<sub>1-62</sub> fused to HopZ1b<sub>59-240</sub> and then HopZ1a<sub>245-369</sub>; HopZ1b1a1b-1 has HopZ1b<sub>1-133</sub> fused to HopZ1a<sub>138-203</sub> and then HopZ1b<sub>200-368</sub>; HopZ1b1a1b-2 has HopZ1b<sub>1-133</sub> fused to HopZ1a<sub>138-244</sub> and then HopZ1b<sub>241-368</sub>; HopZ1b1a1b-3 has HopZ1b<sub>1-58</sub> fused to HopZ1a<sub>63-244</sub> and then HopZ1b<sub>241-368</sub>; HopZ1b1a1b-4 has HopZ1b<sub>1-58</sub> fused to HopZ1a<sub>63-136</sub> and then HopZ1b<sub>134-368</sub>; HopZ1b1a1b-5 has HopZ1b<sub>1-58</sub> fused to HopZ1a<sub>63-203</sub> and then HopZ1b<sub>200-368</sub>.

B. HR assay of *PgyBR1Rif-O1* expressing the wild-type and double-chimeric HopZ1 proteins on soybean. The wild-type and chimeric *hopZ1* genes were carried on the plasmid vector pUCP20tk. The assay was carried out as described in Fig. 1B with the empty pUCP20tk vector (V) used as a control.

C. Soybean *in planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing the wild-type and the double-chimeric HopZ1 proteins. Bacterial populations were measured as described in Fig. 1C. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

D. *In planta* bacterial multiplication of *Pseudomonas syringae* pv. *phaseolicola* strain 1302ARif (*Pph1302ARif*, WT) expressing HopZ1a, HopZ1b, HopZ1a1b1a-2 and HopZ1a1b1a-3 in soybean. The empty pUCP20tk vector (V) was used as a control. Bacterial cell suspensions (OD<sub>600</sub> = 0.005) were infiltrated into the unifoliates of 9-day-old soybeans. Colony-forming units were determined 0 and 3 dpi. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

E. HR assay of *PgyBR1Rif-O1* expressing the wild-type and double-chimeric HopZ1 proteins on *N. benthamiana*. The empty pUCP20tk vector (V) was used as a control. The experiments were carried out as described in Fig. 1D. *N. benthamiana* leaves were harvested and photographed 48–72 h post inoculation.

F. *In planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing the wild-type and double-chimeric HopZ1 proteins in *N. benthamiana*. The assay was carried out as described in Fig. 1E. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

G. Immunoblot showing the wild-type and double-chimeric HopZ1 protein expression in *PgyBR1Rif-O1*. *PgyBR1Rif-O1* cells expressing HopZ1 proteins were induced as described in Fig. 1F. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by anti-HA antibody conjugated with HRP.

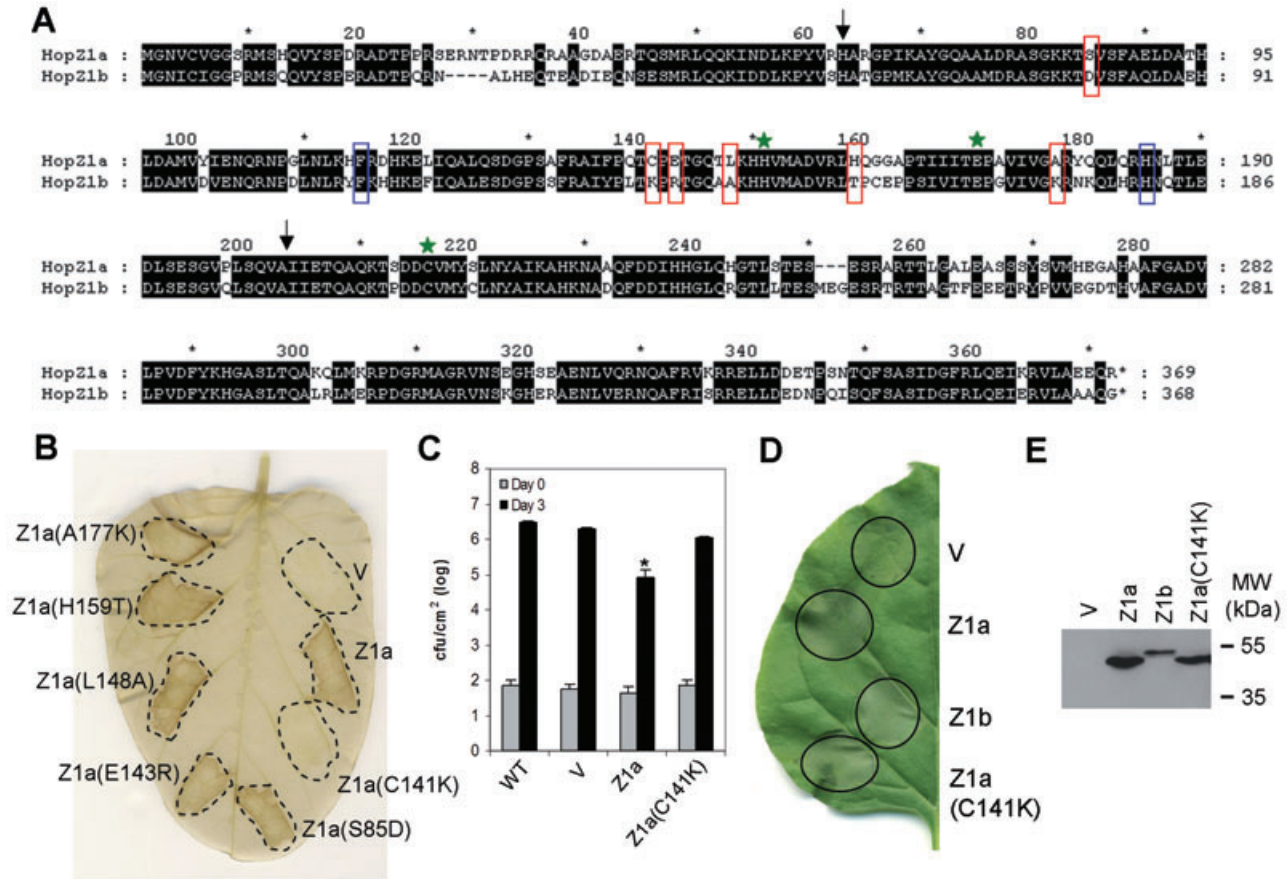
and *in planta* bacterial multiplication assays (Fig. 2F). As expected, all the chimeras triggered HR in *N. benthamiana* (Fig. 2E). *PgyBR1Rif-O1* expressing HopZ1a1b1a-1, HopZ1a1b1a-2, HopZ1a1b1a-3 and HopZ1b1a1b-4 multiplied to a similar level as *PgyBR1Rif-O1* expressing HopZ1b (Fig. 2F). The bacterial populations of *PgyBR1Rif-O1* expressing HopZ1b1a1b-3 and HopZ1b1a1b-5 resembled that of *PgyBR1Rif-O1* expressing HopZ1a (Fig. 2F). *PgyBR1Rif-O1* expressing HopZ1b1a1b-1 and HopZ1b1a1b-2 multiplied in *N. benthamiana* to a level that was higher than *PgyBR1Rif-O1* expressing HopZ1a, but lower than *PgyBR1Rif-O1* expressing HopZ1b (Fig. 2F). It is possible that these chimeric proteins have partial activities of HopZ1a and HopZ1b. Therefore, their phenotypes partially resemble HopZ1a and HopZ1b.

Consistent with the data obtained from the soybean assays, HopZ1a1b1a-2 and HopZ1a1b1a-3 performed exactly like HopZ1b; and HopZ1b1a1b-3 and HopZ1b1a1b-5 performed exactly like HopZ1a (Fig. 2F). These data demonstrated that HopZ1a<sub>63-203</sub> and the corresponding HopZ1b<sub>59-199</sub> determined the HopZ1 allelic specificity in both soybean and *N. benthamiana*. Interestingly, HopZ1a1b1a-1, carrying the region HopZ1b<sub>134-199</sub>, exhibited a HopZ1b-like phenotype (Fig. 2F), suggesting that HopZ1b<sub>134-199</sub> was essential for HopZ1b specific HR-elicitation in *N. benthamiana*. Furthermore, HopZ1b1a1b-4, carrying HopZ1a<sub>63-137</sub>, also showed a HopZ1b-like phenotype (Fig. 2F), indicating that

HopZ1a<sub>63-137</sub> was not sufficient to switch the allele-specific HR elicitation in *N. benthamiana*. The partial activity of HopZ1b1a1b-1, which carries the region HopZ1a<sub>138-203</sub>, suggests that this region itself is also not completely sufficient to confer HopZ1a specific HR-elicitation in *N. benthamiana*. These results from the *N. benthamiana* assays agreed with the data from the soybean assays that HopZ1a<sub>138-203</sub> and HopZ1a<sub>63-137</sub> were both required for triggering HR; and only the region HopZ1a<sub>63-203</sub> was fully sufficient for HopZ1a-specific HR-elicitation.

#### *C141K is a key amino acid change for HopZ1 to evade plant recognition in soybean*

Hypersensitive response assays and *in planta* bacterial multiplication assays in soybean and *N. benthamiana* suggest that the region HopZ1a<sub>63-203</sub> and the corresponding sequence HopZ1b<sub>59-199</sub> determined the allelic specificity of HopZ1. We next identified the key amino acid changes within these regions that confer the alteration in host recognition by site-directed mutagenesis. The amino acid sequence of HopZ1a<sub>63-203</sub> is highly similar to HopZ1b<sub>59-199</sub> with 26 residues different in their biochemical properties (Fig. 3A). Among them, six residues showed high nonsynonymous over synonymous substitution rates by using PAML selection analysis (Ma *et al.*, 2006). PAML is a maximum likelihood-based analysis evaluating the likelihood of different selection models in order to identify specifically selected codons (Yang,



**Fig. 3.** Substitution of cysteine 141 in HopZ1a with a lysine, as in the corresponding position of HopZ1b, is a key mutational change to evade recognition in soybean.

**A.** Amino acid alignment of the full-length sequence of HopZ1a and HopZ1b. Identical and similar residues between the two alleles are shaded. Arrows indicate the borders of the central domain (HopZ1a<sub>63–203</sub> and HopZ1b<sub>59–199</sub>), which determines the HopZ1 allelic specificity. Positions in this domain that are positively selected are framed in red boxes. Residues that are later found to be involved in HopZ1 allelic specificity are framed in blue boxes. Green stars indicate the conserved catalytic triads.

**B.** Soybean HR assay of *PgyBR1Rif-O1* expressing HopZ1a, HopZ1b and HopZ1a site-directed mutants carried on the plasmid vector pUCP20tk. The empty pUCP20tk vector (V) was used as a control. The assay was carried out as described in Fig. 1B. Soybean leaves were harvested 48 h post inoculation and photographed after ethanol bleaching. The experiments were repeated three times with similar results.

**C.** *In planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing HopZ1a and HopZ1a(C141K) in soybean. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. The experiments were repeated three times and data from one experiment are shown. Values that were statistically different are labelled with an asterisk.

**D.** HR assay of *PgyBR1Rif-O1* expressing HopZ1a, HopZ1b and HopZ1a(C141K) in *N. benthamiana*. The empty pUCP20tk vector (V) was used as a control. The assay was carried out as described in Fig. 1D. *N. benthamiana* leaves were harvested and photographed 48–72 h post inoculation. The experiment was repeated three times with similar results.

**E.** Immunoblot showing the protein expression of the wild-type HopZ1 and HopZ1a(C141K) in *PgyBR1Rif-O1*. *PgyBR1Rif-O1* strains were induced as described in Fig. 1F. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by anti-HA antibody conjugated with HRP.

1997). Therefore, it is a powerful tool for understanding the mechanisms of gene evolution (Yang and Nielsen, 2000). High nonsynonymous versus synonymous substitution rates indicate a high likelihood of positive selection acting on certain positions in the sequences, suggesting that these mutations may confer fitness advantage to the individuals carrying them. For example, positive selection occurs when the mutation allows a pathogen to switch from avirulence to virulence in the presence of the corresponding host R gene (Stukenbrock and McDonald,

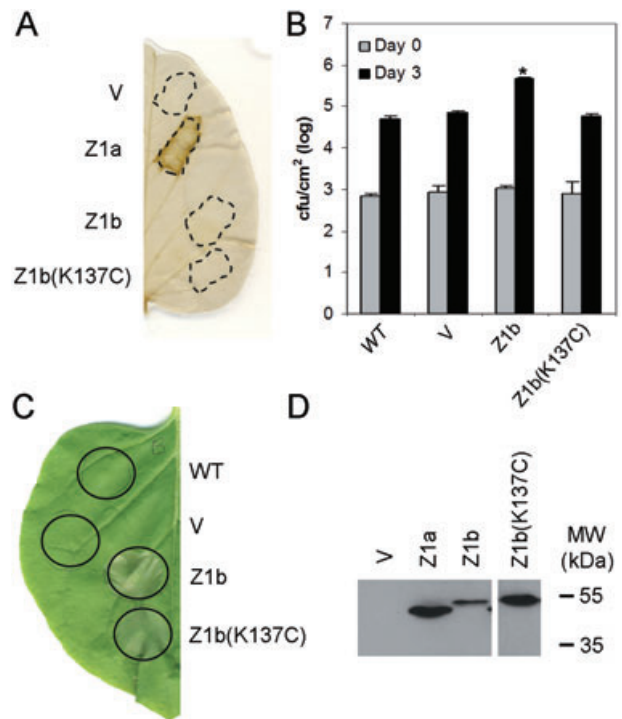
2009). On the contrary, positions that are variable but are not positively selected indicate that these residues may have relaxed selective constraints acting on them. Therefore, these positively selected residues are good candidates that allow HopZ1b to evade host recognition in soybean and may play important roles in determining the allelic specificity. Remarkably, five out of these six positively selected residues, C141, E143, L148, H159 and A177, are in the close proximity to the predicted catalytic residues H151 and E170 (Fig. 3A), indicating that they

may have an impact on the enzymatic characterizations of HopZ1.

To test whether any of the positively selected residues is involved in determining HopZ1 allelic specificity, six HopZ1a single mutants were constructed with each of the residues replaced by the amino acid in the corresponding position in HopZ1b. pUCP20tk carrying these mutated HopZ1a genes were electroporated into *PgyBR1Rif-O1* and their HR-eliciting activities in soybean were examined. Among these HopZ1a mutants, only HopZ1a(C141K) was no longer able to elicit an HR in soybean (Fig. 3B). Consistent with the HR assay, *PgyBR1Rif-O1* expressing HopZ1a(C141K) multiplied to a similar level as *PgyBR1Rif-O1* carrying the empty vector, whereas *PgyBR1Rif-O1* expressing the wild-type HopZ1a showed a significant decrease in bacterial multiplication in soybean (Fig. 3C). This result confirms that a single substitution of C141 with a lysine, as in the corresponding position (K137) in HopZ1b, abolished the HR-eliciting activity of HopZ1a.

It has been previously shown that the mutant HopZ1a(C216A), with the catalytic cysteine216 substituted by an alanine, lost HR-eliciting activity in soybean, *Arabidopsis* and *N. benthamiana* (Ma *et al.*, 2006; Lewis *et al.*, 2008; Zhou *et al.*, 2009). These data indicate that the enzymatic activity is required for HopZ1a to trigger HR in plant hosts. Therefore, it is possible that HopZ1a(C141K) lost its HR-eliciting activity due to the loss of enzymatic activity. To test this possibility, we inoculated *N. benthamiana* with *PgyBR1Rif-O1* expressing HopZ1a(C141K). *PgyBR1Rif-O1* expressing HopZ1a(C141K) still triggered HR in *N. benthamiana* (Fig. 3D), suggesting that the cysteine141 to lysine substitution did not seem to abolish the enzymatic activity of HopZ1a. Therefore, the deficiency of HR elicitation of HopZ1a(C141K) in soybean is unlikely due to the loss of enzymatic activity.

We then constructed a HopZ1b mutant, HopZ1b(K137C), with the amino acid residue K137 replaced by a cysteine and examined the phenotype of this mutant in soybean. HopZ1b(K137C) carried on pUCP20tk was expressed in *PgyBR1Rif-O1* under the control of the *hopZ1b* promoter. *PgyBR1Rif-O1* expressing HopZ1b(K137C) did not gain the ability to trigger HR in soybean (Fig. 4A), suggesting that the replacement of K137 with a cysteine is not sufficient for HopZ1b to be recognized in soybean. This result is consistent with the domain shuffling results, showing that the region HopZ1a<sub>63-137</sub> is also required for HR elicitation. We next examined whether HopZ1b(K137C) still exhibited the non-host growth promotion activity by measuring the bacterial population of *Pph1302ARif* expressing HopZ1b(K137C) in soybean. In contrast to the wild-type HopZ1b, *Pph1302ARif* expressing HopZ1b(K137C) multiplied to a similar level with *Pph1302ARif* carrying the



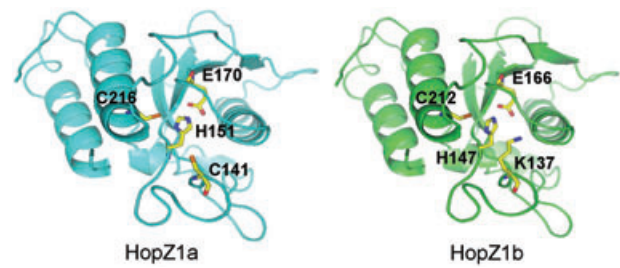
**Fig. 4.** Virulence activity and HR-elicitation activity of HopZ1b(K137C) on soybean and *N. benthamiana*. All the experiments were repeated three times with similar results. Data from one experiment are shown. A. Soybean HR assay of *PgyBR1Rif-O1* expressing HopZ1a, HopZ1b and HopZ1b(K137C) carried on the plasmid vector pUCP20tk. The empty pUCP20tk vector (V) was used as a control. The assay was carried out as described in Fig. 1B. Inoculated leaves were harvested 48 h post inoculation and photographed after ethanol bleaching. B. Effect of HopZ1b(K137C) mutants on the *in planta* bacterial growth of *Pph1302ARif* in soybean. *In planta* bacterial populations of *Pph1302ARif* (WT) expressing HopZ1b and HopZ1b(K137C) were measured as described in Fig. 2D. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with an asterisk. C. HR assay of *PgyBR1Rif-O1* expressing HopZ1b and HopZ1b(K137C) in *N. benthamiana*. *PgyBR1Rif-O1* (WT) and *PgyBR1Rif-O1* carrying the empty pUCP20tk vector (V) were used as controls. The assay was carried out as described in Fig. 1D. *N. benthamiana* leaves were harvested and photographed 48–72 h post inoculation. D. Immunoblot showing the protein expression of the wild-type HopZ1 and HopZ1b(K137C) in *Pph1302ARif*. *Pph1302ARif* strains were induced as described in Fig. 1F. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by anti-HA antibody conjugated with HRP.

empty vector (Fig. 4B), demonstrating that this mutant was no longer able to promote *Pph1302ARif* multiplication in soybean. We also confirmed that HopZ1b(K137C) protein expressed in a similar level in *Pph1302A* as the wild-type HopZ1b (Fig. 4D). Therefore, the loss of the virulence function of this mutant was not due to the lack of protein expression in *P. syringae*.

HopZ1b triggers HR in *N. benthamiana*. Similar to HopZ1a, the catalytic mutant HopZ1b(C212A), with the catalytic cysteine212 substituted by an alanine, also loses HR-eliciting activity (Zhou *et al.*, 2009), indicating that the enzymatic activity is required for HopZ1b to trigger HR in *N. benthamiana*. To test the possibility that HopZ1b(K137C) lost its virulence activity due to the loss of enzymatic activity, we inoculated *N. benthamiana* with PgyBR1Rif-O1 expressing HopZ1b(K137C). PgyBR1Rif-O1 expressing HopZ1b(K137C) was still able to induce HR in *N. benthamiana* (Fig. 4C), suggesting that the loss of growth promoting activity of HopZ1b(K137C) is unlikely due to the loss of enzymatic activity.

#### C141/K137 may be involved in substrate-binding specificity of HopZ1

Substitution analysis on the C141 of HopZ1a and the K137 in the corresponding position of HopZ1b suggests that the amino acid residue at this position is essential for the allelic specificity of HopZ1. To understand the underlying mechanism, we analysed the C141/K137 residue in the spatial structures of HopZ1. So far, no protein structure is available for proteins belonging to the YopJ family or the C55 cysteine protease family. Therefore, we conducted homology modelling analysis and generated structural models for HopZ1 proteins using the structure of XopD, a T3SE secreted by the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*, as a template. XopD belongs to the C48 cysteine protease family (Rawlings *et al.*, 2006), and desumoylates the small ubiquitin-like modifier (SUMO) substrates *in planta* (Hotson *et al.*, 2003; Chosed *et al.*, 2007). The structure of XopD is the best match that could be found in the Protein Data Bank. The secondary structure prediction and comparative analysis showed that the predicted secondary structures of HopZ1 catalytic cores were similar to the secondary structure of the catalytic core of XopD defined by its crystal structure. Structure modelling suggested that the C141 residue of HopZ1a was located on a flexible loop structure (Q139–K149) close to the entrance of the catalytic pocket consisting the conserved catalytic residues H151, E170 and C216 (Fig. 5). In HopZ1b, a lysine (K137) replaces the cysteine at this position. Lysine is a long and positively charged residue, while cysteine is a hydrophobic residue with a highly reactive thiol side chain. It is tempting to hypothesize that the residue in this position may directly interact with or mediate the interactions of HopZ1 with host substrate(s) *in planta*. The significant difference in the biochemical characteristics of lysine and cysteine indicates that the mutation of C141 in a HopZ1a-like ancestral allele to a lysine may have changed HopZ1 interactions with host substrates in a way that the newly evolved alleles no longer elicit defence responses. In



**Fig. 5.** Structural models of the catalytic domains of hopZ1a (left) and hopZ1b (right). The conserved catalytic residues (H151, E170 and C216 in HopZ1a; H147, E166 and C212 in HopZ1b) and the key residue C141/K137 near the active site are labelled and shown as bond models.

addition, HopZ1b(K137C) loses the pathogen multiplication promoting activity in soybean, suggesting that the C141K substitution may also allow the newly evolved allele to gain novel virulence activity, possibly by allowing HopZ1b to interact with new host substrates.

Taken together, these data suggest that the C141/K137 residue is important in determining the functional diversification of HopZ1 alleles, probably by mediating allele-specific substrate binding in soybean. Since this position is under strong positive selection, the C141/K137 substitution may be a key mutational change for HopZ1b to evade plant recognition and retain virulence function during the co-evolutionary arms race.

#### F115/F111 and H185/H181 mediate HopZ1 allelic specific HR elicitation in *N. benthamiana*

In a previous study characterizing the HR-eliciting activity of HopZ1a and HopZ1b in *N. benthamiana*, we reported that although these two alleles both induced cell death symptoms, they appeared to trigger different resistance pathways in *N. benthamiana*. These data indicate that differentiated resistance systems have evolved in *N. benthamiana* to adapt to HopZ1 allelic diversification (Zhou *et al.*, 2009). Taking advantage of this pathosystem, we took a random mutagenesis approach to identify additional residues involved in HopZ1 allelic specificity by identifying residues required for HopZ1b-triggered, but not HopZ1a-triggered HR in *N. benthamiana*.

PCR-based random mutagenesis was performed according to Vartanian *et al.* (1996). PCR products, most of which contain 1–3 mutations throughout the full-length HopZ1b, were ligated into pUCP20tk downstream of the *hopZ1b* promoter. This mutant library was electroporated into PgyBR1Rif-O1 and individual clones were screened by infiltrating *N. benthamiana* leaves for the mutants deficient in triggering HR. Among the 264 mutagenized clones examined, 36 were found to be defective in HR elicitation. These mutants were sequenced in order to identify the mutation sites. Sequencing data showed that

most of the mutants had nonsense mutations or substitutions occurring in the catalytic triad, which were known to be required for HR elicitation in *N. benthamiana* (Zhou *et al.*, 2009). Two novel sites, F111 and H181, were identified as candidate residues critical for HopZ1b to trigger HR in *N. benthamiana*. Interestingly, these two residues are within the region HopZ1b<sub>59–199</sub>, corresponding to HopZ1a<sub>63–203</sub>, which was identified as the essential domain for HopZ1 allelic specificity from the domain shuffling experiments. These residues were further analysed using alanine substitution mutagenesis. The mutant genes *hopZ1b(F111A)* and *hopZ1b(H181A)* were introduced into the vector pUCP20tk, and the recombinant plasmids were electroporated into *PgyBR1Rif-O1*. Infiltration of *PgyBR1Rif-O1* expressing HopZ1b(F111A) or HopZ1b(H181A) in *N. benthamiana* revealed that neither of the mutants elicited HR symptoms (Fig. 6A). Furthermore, bacterial population of *PgyBR1Rif-O1* expressing HopZ1b(F111A) or HopZ1b(H181A) was similar to that of *PgyBR1Rif-O1* harbouring the empty vector in *N. benthamiana* and significantly higher than that of *PgyBR1Rif-O1* expressing HopZ1b (Fig. 6B). The bacterial multiplication assays again confirmed that both mutants lost HR-triggering activity in *N. benthamiana*.

Data from the HR assays and the *in planta* bacterial multiplication assays confirmed that F111 and H181 were required for HopZ1b to trigger HR in *N. benthamiana*. The normal protein expression of the mutated genes in *PgyBR1Rif-O1* induced by the M63 minimal medium was verified using Western blots, confirming that the loss of HR elicitation of the mutants was not due to the alteration in protein expression (Fig. 6F).

Since F111 and H181 are conserved in HopZ1 alleles, we then analysed the role of the corresponding F115 and H185 residues in HopZ1a-triggered HR in *N. benthamiana*. Substitution mutants *hopZ1a(F115A)* and *hopZ1a(H185A)* were constructed and ligated to pUCP20tk, and the recombinant plasmids were electroporated into *PgyBR1Rif-O1*. Interestingly, *PgyBR1Rif-O1* expressing HopZ1a(F115A) or HopZ1a(H185A) still triggered HR in *N. benthamiana*, similar to the wild-type HopZ1a (Fig. 6A and B). These data suggested that unlike HopZ1b, the phenylalanine and histidine residues were not required for HR-elicitation of HopZ1a in *N. benthamiana*.

We then examined the role of F115/F111 and H185/H181 in the HR elicitation activity of HopZ1a and the virulence activity of HopZ1b in soybean. Substitution of F115 or H185 with an alanine abolished the HR elicitation of HopZ1a in soybean, evidenced by the lack of cell death symptoms in the leaf areas infiltrated with *PgyBR1Rif-O1* expressing HopZ1a(F115A) or HopZ1a(H185A) (Fig. 6C). Consistent with the HR assay, *PgyBR1Rif-O1* expressing HopZ1a(F115A) or HopZ1a(H185A) multiplied to the

same level as *PgyBR1Rif-O1* harbouring the empty vector in soybean (Fig. 6D), suggesting that F115 and H185 were required for HR elicitation of HopZ1a in soybean.

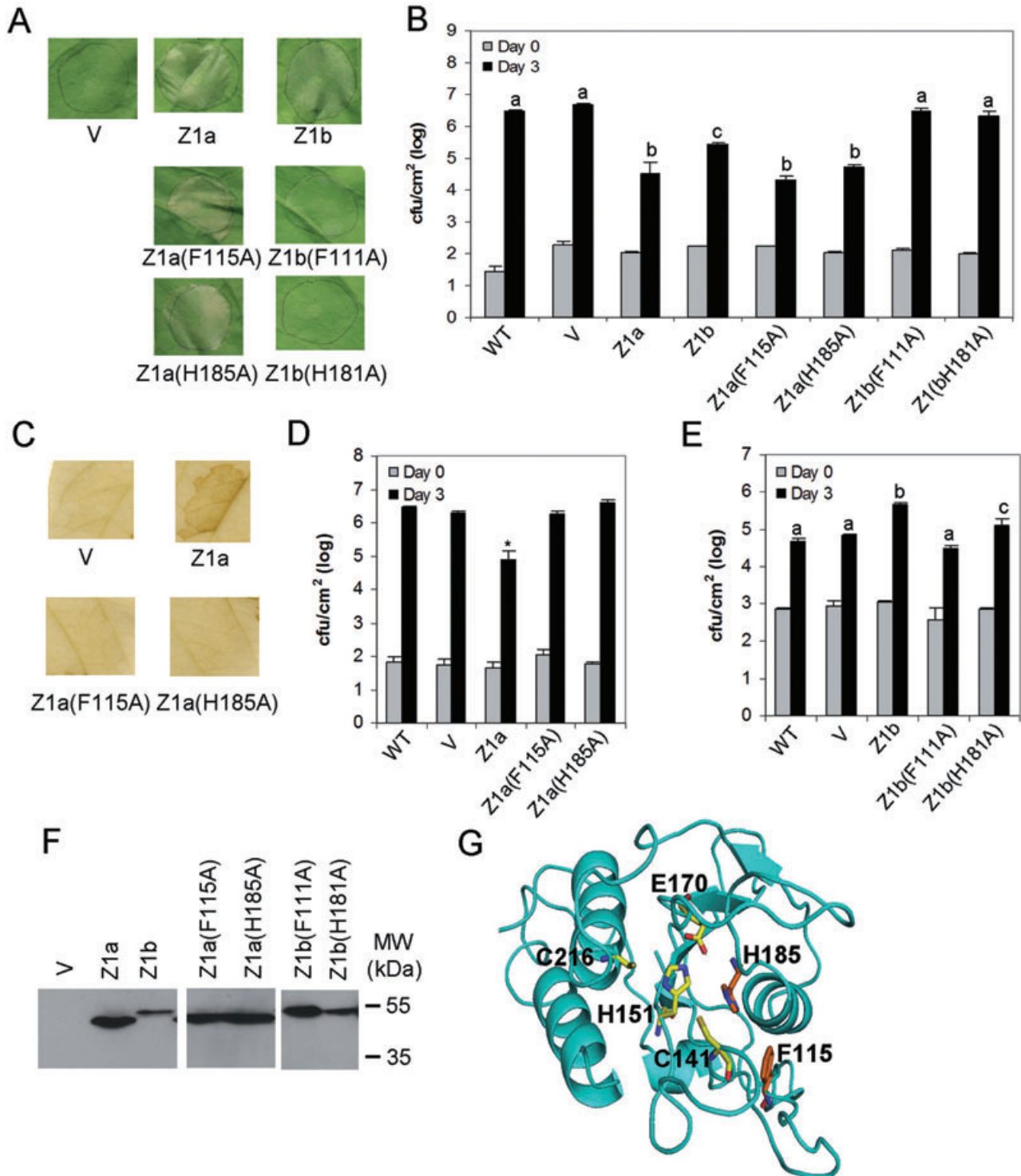
The mutants HopZ1b(F111A) and HopZ1b(H181A) were also evaluated for their virulence activity by measuring the levels of bacterial growth (Fig. 6E). After infiltration, the bacterial population of *Pph1302ARif* expressing HopZ1b(F111A) was not different from that of *Pph1302ARif* carrying the empty vector, demonstrating that HopZ1b(F111A) was no longer able to promote *Pph1302ARif* multiplication in soybean. The bacterial population of *Pph1302ARif* expressing HopZ1b(H181A) was lower than that of *Pph1302ARif* expressing the wild-type HopZ1b, but higher than *Pph1302ARif* carrying the empty vector, indicating that the mutation of H181 had a negative effect on the virulence function of HopZ1b in soybean. It is likely that these residues are evolutionary maintained because mutations in either of the two residues impair the virulence activity in HopZ1b, although their mutations would lead to evasion of host recognition in soybean.

Homology modelling using XopD protein structure as a reference suggests that both residues are located on flexible loop structures (Fig. 6G). The H185/H181 residue appears to be close to the catalytic histidine residue H151/H147 and the active site. And the F115/F111 residue seems to be in the close proximity of both the C141/K137 loop region and the H185/H181 loop region. Therefore, the F115/F111 and H185/H181 residues could be involved in maintaining the protein conformation and/or affecting the binding of certain substrates to the active site.

#### A model for HopZ1 allelic specificity

HopZ1 is an ancestral T3SE produced by *P. syringae* with three allelic forms evolved through pathoadaptation (Ma *et al.*, 2006). These allelic forms exhibit distinct cellular functions *in planta* (Zhou *et al.*, 2009). Among them, HopZ1a most assembles the ancestral form and induces defence responses in soybean and *N. benthamiana*. Interestingly, HopZ1b, derived from a HopZ1a-like ancestor, evades the host recognition in soybean. The high similarity between full-length HopZ1a and HopZ1b sequences allowed us to conduct domain shuffling and mutagenesis studies in order to understand the molecular mechanisms underlying the allelic specificity of these HopZ1 alleles.

HopZ1 diversification is likely driven by the plant R gene-imposed selective pressure. R proteins are usually evolved to recognize structural or enzymatic features of T3SEs (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). So far, very few T3SEs are found to directly interact with their matching R proteins in plants (Deslan-



des *et al.*, 2003). On the contrary, it is believed that many R proteins have evolved to indirectly recognize their cognate effectors by monitoring (or guarding) the modification of the effector targets (Dangl and Jones, 2001; van der Hoorn *et al.*, 2002). HopZ1 alleles have a conserved catalytic triad that is identical to that of the C55 family of

cysteine protease. However, a homologous T3SE in *Yersinia* spp., YopJ, exhibits an acetyltransferase activity, which requires the same catalytic triad (Mittal *et al.*, 2006; Mukherjee *et al.*, 2006). Prior experiments clearly showed that the catalytic mutants of HopZ1a and HopZ1b were abrogated in their HR-eliciting activity and virulence func-

**Fig. 6.** Virulence activities and HR-elicitation activities of the H111/F111 and H185/H181 mutants. All the experiments were repeated three times with similar results. Data from one experiment are shown.

A. HR assay of *PgyBR1Rif-O1* expressing HopZ1a, HopZ1b, HopZ1a(F115A), HopZ1a(H185A), HopZ1b(F115A) and HopZ1b(H185A) on *N. benthamiana*. The empty pUCP20tk vector (V) was used as a control. The assay was carried out as described in Fig. 3D. *N. benthamiana* leaves were harvested and photographed 48 h post inoculation.

B. *In planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing the wild-type and mutant HopZ1 in *N. benthamiana*. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

C. Soybean HR assay of *PgyBR1Rif-O1* expressing HopZ1a, HopZ1a(F115A) and HopZ1a(H185A). The empty pUCP20tk vector (V) was used as a control. Soybean leaves were harvested 48 h post inoculation and photographed after ethanol bleaching.

D. *In planta* bacterial multiplication of *PgyBR1Rif-O1* (WT) expressing HopZ1a, HopZ1a(F115A) and HopZ1a(H185A) in soybean. The empty pUCP20tk vector (V) was used as a control. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with an asterisk.

E. *In planta* bacterial multiplication of *Pph1302ARif* (WT) expressing HopZ1b, HopZ1b(F111A) and HopZ1b(H181A) in soybean. The empty pUCP20tk vector (V) was used as a control. The assay was carried out as described in Fig. 2D. Each value represents the mean of bacterial growth in four leaves. Error bars represent the standard errors. Values that were statistically different are labelled with different letters.

F. Immunoblot showing the protein expression of the wild-type and mutant HopZ1 in *Pph1302ARif*. *Pgy1302ARif* cells expressing HopZ1 proteins were induced in M63 minimal medium containing 1% fructose overnight at room temperature. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by anti-HA antibody conjugated with HRP.

G. Homology modelling of the catalytic domain of HopZ1a. Structural model of the HopZ1a catalytic core domain showing the conserved catalytic residues (H151, E170 and C216) and the nearby residues F115, C141 and H185.

tions, indicating that the enzymatic activity was essential for the cellular functions of HopZ1. It is also worth noting that the catalytic residues of other YopJ family members, such as YopJ (Orth *et al.*, 2000), AvrBsT (Orth *et al.*, 2000), AvrXv4 (Roden *et al.*, 2004), AvrRxv (Bonshtien *et al.*, 2005), HopZ2 (Ma *et al.*, 2006; Lewis *et al.*, 2008) and HopZ3 (Zhou *et al.*, 2009), are also required for their functions. Therefore, it is likely that the host recognition of HopZ1a is mediated by the modification of certain HopZ1a substrate(s) in soybean.

Domain shuffling experiments and the C141/K137 residue exchange analysis support a hypothesis that HopZ1 allelic specificity may depend on allele-distinctive substrate-binding activities. We determined that the region HopZ1a<sub>63–203</sub> and its corresponding region HopZ1b<sub>59–199</sub> were responsible for HopZ1 allelic specific host responses. This central domain contains the first two catalytic residues (H151 and E170 in HopZ1a, and H147 and E166 in HopZ1b) and is immediately upstream of the catalytic cysteine residue (C216 in HopZ1a, and C212 in HopZ1b). We suspect that this region may be involved in maintaining HopZ1 protein conformation and/or binding to specific substrates, which then leads to allelic specificity. Furthermore, the C141/K137 residue may have direct contact with specific substrate(s) of HopZ1a or HopZ1b respectively.

The identification of two additional residues, the conserved phenylalanine (F115/F111) and the histidine (H185/H181), which are involved in the allelic specificity of HopZ1, also supports the model that HopZ1a and HopZ1b trigger allelic specific defence responses in *N. benthamiana* by interacting with allele-specific substrates. HopZ1a and HopZ1b trigger distinct defence pathways in *N. benthamiana*. The F111 and H181 residues are required for HopZ1b-triggered HR, probably by mediating HopZ1b interaction with certain host protein(s) in *N. benthamiana*.

However, the conserved F115 and H185 residues in the corresponding positions of HopZ1a are not required for HopZ1a-triggered HR, indicating that HopZ1a-triggered HR may involve HopZ1a interactions with different host protein(s) in *N. benthamiana* and may require other residues. These results are also consistent with our previous observations that the conserved N-terminal glycine (G2) is required for HopZ1b-triggered HR, but not HopZ1a-triggered HR in *N. benthamiana* (Zhou *et al.*, 2009). HopZ1 proteins are mainly located on the plasma membranes *in planta* and the residue G2 is likely involved in the myristylation-mediated membrane localization of HopZ1 (Lewis *et al.*, 2008; Zhou *et al.*, 2009). HopZ1a(G2A) still triggers HR in *N. benthamiana* although it loses its membrane localization. However, HopZ1b(G2A) no longer triggers HR in *N. benthamiana*, suggesting that HopZ1b-triggered HR may be dependent on its membrane localization (Zhou *et al.*, 2009). Together with the new data reported in this paper, it is possible that HopZ1a- and HopZ1b-triggered HR in *N. benthamiana* involves their interactions with different host substrates, which may be located in different cellular compartments.

Since the conserved F115/F111 and H185/H181 residues did not impact the HR-elicitation of HopZ1a in *N. benthamiana*, it is likely that they are not directly required for the enzymatic activity of HopZ1. Rather, they seem to be involved in mediating HopZ1 interactions with specific plant substrate(s). However, with the current evidence, we cannot rule out the possibility that H181 and F111 in HopZ1b may still be important for the enzymatic activity of HopZ1b, even when the corresponding H185 and F115 seem not to be critical for the enzymatic activity of HopZ1a. For example, the H181 and F111 residues may be involved in the maintenance of the overall structure of the catalytic domain in HopZ1b. This possibility can only be tested after the development of an assay evaluating

the *in planta* enzymatic activity of HopZ1 effectors, which is not available at this time.

It is noteworthy that although F115 and H185 residues are not critical for HopZ1a-triggered HR in *N. benthamiana*, they are required for HR elicitation of HopZ1a in soybean. There are three possibilities to explain these data: (i) HR elicitation of HopZ1a requires its interactions with different host substrates in soybean and *N. benthamiana*; (ii) HR elicitation of HopZ1a in soybean and *N. benthamiana* involves its interactions with homologous proteins; but the sequence difference between these homologues in different plant species may result in the requirement of direct contacts with different HopZ1a residues; (iii) HopZ1a substrate(s) are guarded by different R proteins in soybean and *N. benthamiana*.

Collectively, our data suggest that HopZ1a and HopZ1b interact with different sets of substrates in plant hosts. Many T3SEs have multiple targets in the host (van der Hoorn and Kamoun, 2008; Hogenhout *et al.*, 2009). We hypothesize that some substrates interact with both HopZ1a and HopZ1b, but some others are allele-specific substrates. These allele-specific substrates, requiring distinct HopZ1 residues for protein-protein interaction, determine the allelic specificity of HopZ1. To test this hypothesis, we started yeast two-hybrid screens to identify HopZ1-interacting proteins from soybean using HopZ1a and HopZ1b as the baits respectively. Our preliminary results revealed five proteins interacting with both HopZ1a and HopZ1b in yeast. Interestingly, three soybean proteins only interacted with HopZ1a and one protein only interacted with HopZ1b (H. Zhou and W. Ma, unpubl. data). These preliminary data strongly supported our hypothesis.

### Conclusions

A co-evolutionary arms race leads to escalatory adaptations, where adaptations in pathogens and hosts occur in a stepwise manner. The zigzag model elegantly demonstrated that changes in the complement of pathogen T3SEs or host defence-related proteins can dramatically shift the balance of power between plants and pathogens. Despite the essential role that the co-evolutionary arms race plays in the dynamics and outcomes of pathogen–host interactions, we are just beginning to gather information in order to reconstruct some of these dynamic evolutionary processes.

The evolutionarily conserved and diversified HopZ1 effectors provide a useful model for the study of the stepwise effector co-evolution with plant immunity. In this study, we are able to trace the history of the HopZ1 evolution. Our data highlight the importance of the catalytic domain in HopZ1 allelic specificity. The identification of amino acid residues that are essential for the switch of the substrate-binding specificities between HopZ1a and

HopZ1b, especially the C141/K137 residue, further suggests that this substitution is critical for the HopZ1a-like ancestral allele to evade host recognition. Furthermore, the C141/K137 mutation may also allow HopZ1b to maintain its virulence function through the establishment of novel interactions with new substrate(s) in soybean. Considering that the C141/K137 residue has a high likelihood to be positively selected, this substitution might be a key mutational change during the arms race with the plant hosts that drove the evolution of HopZ1 alleles.

The evolution of HopZ1b again imposes selective pressures on the plant hosts. We present evidence showing that the conserved F115/F111 and H185/H181 residues contribute differently to HR elicitation by HopZ1a and HopZ1b in *N. benthamiana*, suggesting that different host substrates may be involved in the HopZ1a-triggered and HopZ1b-triggered HR in *N. benthamiana*. These data indicate that *N. benthamiana* may have evolved a new defence mechanism, which is different from the one recognizing the HopZ1a-like ancestor, to recognize the newly evolved HopZ1b. We propose to designate the HopZ1b-triggered immunity in *N. benthamiana* as a second tier ETI, which is distinct from the HopZ1a-triggered first tier ETI.

Our previous and present data lead to a hypothesis that sequence diversification-enabled HopZ1 evolution allowed altered substrate-binding specificity of HopZ1 alleles in plant hosts in a way that the newly evolved allele, HopZ1b, is no longer recognized by the host while is still able to promote pathogen infection. Future characterization of the HopZ1-interacting proteins from the plant hosts, such as soybean, will help test this hypothesis and address interesting questions that remain unanswered in this system, such as the mechanisms underlying the HR-elicitation activity of HopZ1a and the virulence function of HopZ1b. The mutants isolated and constructed in this study will be useful tools in defining the basis of the allele-specific substrate interactions of HopZ1.

### Experimental procedures

#### Bacterial strains and plasmids

*Pseudomonas syringae* strains were grown at 30°C in King's B medium (King *et al.*, 1954) or M63 minimal medium containing 1% fructose for the induction of T3SS (Bretz and Hutcheson, 2004). Plasmids were introduced into *P. syringae* by electroporation using an Eppendorf electroporator 2510 (Eppendorf North America, Westbury, NY, USA) according to the manufacturer's instructions. *Agrobacterium tumefaciens* and *Escherichia coli* were grown in Luria–Bertani medium (BD Co., Franklin Lakes, NJ, USA) at 30°C or 37°C respectively. Transformation of plasmids to *A. tumefaciens* and *E. coli* were accomplished by introducing plasmid DNA to chemical competent cells as previously described (Sambrook and Russell, 2001). Appropriate antibiotics were supplemented to the media when necessary.

Antibiotics were used at the following concentrations ( $\mu\text{g ml}^{-1}$ ): kanamycin, 50; rifampicin, 50; tetracycline, 5.

Bacteria strains and plasmids used in this study are summarized in Table 1.

### Gene cloning and plasmid construction

PCR-based random mutagenesis was used to generate a mutant library of *hopZ1b* genes using the manganese and unbalanced dNTP method (Vartanian *et al.*, 1996). The *hopZ1b* gene was amplified using 0.5 mM  $\text{MnCl}_2$ , 300  $\mu\text{M}$  dCTP, 300  $\mu\text{M}$  dATP, 1000  $\mu\text{M}$  dTTP and 1000  $\mu\text{M}$  dGTP. The PCR products were then ligated into the broad host-range plasmid vector pUCP20tk. Site-directed mutagenesis was accomplished by introducing the mutations into PCR primers used to amplify the genes. The mutated and the chimeric *hopZ1* genes were cloned downstream of the native promoters (including the *hrp* box) of the wild-type genes in pUCP20tk. All the proteins were tagged with an in-frame haemagglutinin (HA) at the C-termini.

For *Agrobacterium*-mediated transient expression, the wild-type and chimeric *hopZ1* genes were cloned into a binary vector pMDD1 under the control of cauliflower mosaic virus (CaMV) 35 s promoter (Mudgett *et al.*, 2000). All proteins were tagged with HA at the C-termini. The resulting plasmids were then transformed into *A. tumefaciens* C58C1 (pCH32) by chemical transformation (Van larebeke *et al.*, 1974).

Oligonucleotide sequences used as primers in PCR reactions to generate the recombinant plasmids are available upon request.

### Bacteria growth assays and hypersensitive responses (HR) assay on soybean and *N. benthamiana*

Soybean [*Glycine max* (L.) cv. William 82] seeds were surface sterilized with 10% bleach for 10 min and pre-germinated on wet filter paper at room temperature in the dark for 4 days. The seedlings were then transplanted to soil and grown in a greenhouse at an ambient temperature of 25–30°C under natural light conditions.

For bacterial multiplication assays in soybean, *P. syringae* pv. *glycinea* (*Pgy*) and *P. syringae* pv. *phaseolicola* (*Pph*) expressing the wild-type, mutated and chimeric *hopZ1* genes were grown on King's B agar overnight at 30°C. The cells were scraped from the agar and suspended in 10 mM  $\text{MgSO}_4$  at an  $\text{OD}_{600}$  of 0.0001 for *Pgy* (approximately  $2 \times 10^5$  cfu  $\text{ml}^{-1}$ ) and an  $\text{OD}_{600}$  of 0.005 for *Pph*. Nine-day-old soybean seedlings, with fully developed unifoliates, were infiltrated with the bacterial cell suspensions using a needle-less syringe. Following inoculation, the plants were transferred to a growth chamber with constant 22°C, 90% humidity and a 16 h photoperiod. To determine the *in planta* bacteria populations, four leaf discs (0.5  $\text{cm}^2$ ) were taken from each inoculated leaf, briefly surface sterilized in 70% ethanol and then homogenized in 10 mM  $\text{MgSO}_4$ . Serial dilutions were subsequently plated onto King's B agar with appropriate antibiotics to determine the colony forming units (cfu).

For HR assays in soybean, *P. syringae* cells scraped from the King's B agar were suspended in 5 mM MES (pH5.6) at

an  $\text{OD}_{600}$  of 0.3, and then infiltrated into the unifoliates of 14-day-old soybean seedlings. The infiltrated plants were kept in a growth chamber with high humidity (~90%) for 48 h. Infiltrated leaves were then harvested and bleached in 95% ethanol for a better visualization of the cell death symptoms.

For bacterial multiplication assays in *N. benthamiana*, *Pgy*BR1Rif-O1 strains were grown overnight on King's B agar. Cells scraped from the agar were suspended in 10 mM  $\text{MgSO}_4$  at  $\text{OD}_{600}$  of 0.0001 and used to infiltrate fully expanded leaves of 4-week-old *N. benthamiana* plants. The inoculated plants were kept at room temperature with a 16 h photoperiod. Four leaf discs (0.5  $\text{cm}^2$ ) were taken from each inoculated leaf, briefly surface sterilized in 70% ethanol and then homogenized in 10 mM  $\text{MgSO}_4$ . Serial dilutions were plated onto King's B agar plates with appropriate antibiotics to determine the cfu.

For HR assays in *N. benthamiana*, *P. syringae* cell suspensions in 10 mM  $\text{MgSO}_4$  at  $\text{OD}_{600}$  of 0.01 were used to infiltrate fully expanded leaves of 4-week-old *N. benthamiana* plants. Photos of the infiltrated leaves were taken 72 h post inoculation.

### *Agrobacterium*-mediated transient expression in *N. benthamiana*

*Agrobacterium tumefaciens* strain C58C1 (pCH32) harbouring pMDD1 carrying the chimeric and the wild-type *hopZ1* genes was used to infiltrate 4-week-old *N. benthamiana* leaves using a needle-less syringe at an  $\text{OD}_{600}$  of 0.5 as previously described (Szurek *et al.*, 2001; Ma *et al.*, 2006). Inoculated plants were kept at room temperature under continuous low light. Cell death symptoms were monitored 72 h post inoculation. The empty pMDD1 vector was used as a negative control.

### Protein analysis

*Pseudomonas syringae* expressing the *hopZ1* genes carried on pUCP20tk was grown in King's B medium with kanamycin at 30°C overnight. The bacteria cells were washed once, resuspended in M63 minimal medium supplemented with 1% fructose at an  $\text{OD}_{600}$  of 0.5 and incubated with agitation at room temperature overnight. The induced bacterial cell cultures were normalized to an  $\text{OD}_{600}$  of 0.5, and cells from 1 ml of the induced culture were collected. The induced cells were resuspended in 2 $\times$  Laemmli buffer and then boiled for 5 min (Laemmli, 1970). Total bacteria proteins were separated by SDS-PAGE. HopZ1 expression was detected by Western blots using anti-HA antibody conjugated with Horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Homology modelling analysis

Secondary structure prediction for *hopZ1* proteins was carried out using the Jpred server (Cole *et al.*, 2008). Sequence alignment was performed between *hopZ1* catalytic core domains and XopD catalytic core based on their secondary structure similarity. The comparative modelling

**Table 1.** Bacteria strains and plasmids.

Strains or plasmids	Description	Source/reference
<i>Escherichia coli</i> DH5 $\alpha$	F <sup>-</sup> $\Phi$ 80d/ <i>lacZ</i> ΔM15 Δ( <i>lacZ</i> YA- <i>argF</i> ) U169 <i>recA1 endA1, hsdR17</i> (rk-, mk+) <i>phoA supE44 λ- thi-1 gyrA96 relA1</i>	Invitrogen, Carlsbad, CA
<i>Pseudomonas syringae</i> pv. <i>glycinea</i> strain BR1Rif-O1	Endogenous plasmid (containing <i>hopZ1b</i> ) cured line of <i>Pseudomonas syringae</i> pv. <i>glycinea</i> strain BR1Rif, which was isolated from soybean, Rif <sup>R</sup>	Zhou <i>et al.</i> (2009)
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> strain 1302ARif	Spontaneous rifampicin resistant mutant of <i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> strain 1302A, which was isolated from kidney bean Rif <sup>R</sup> , Tet <sup>R</sup>	Zhou <i>et al.</i> (2009)
<i>Agrobacterium tumefaciens</i> C58C1 (pCH32)		MB Mudgett
pUCP20tk	Plasmid vector used to express proteins in <i>P. syringae</i> , Kan <sup>R</sup>	West <i>et al.</i> (1994); DS Guttman
pUCP20tk::HopZ1-HA	HopZ1 alleles tagged with HA and under the control of their native promoters were inserted in pUCP20tk, Kan <sup>R</sup>	Ma <i>et al.</i> (2006); Lewis <i>et al.</i> (2008)
pUCP20tk::HopZ1a(C216A)-HA	HopZ1a including its native promoter and with cysteine216 replaced with an alanine was inserted in pUCP20tk, Kan <sup>R</sup>	Lewis <i>et al.</i> (2008)
pUCP20tk::HopZ1b(C212A)-HA	HopZ1b including its native promoter and with cysteine212 replaced with an alanine was inserted in pUCP20tk, Kan <sup>R</sup>	Lewis <i>et al.</i> (2008)
pUCP20tk::HopZ1a1b-1-HA	pUCP20tk carrying the native promoter of <i>hopZ1a</i> and a chimeric protein with HopZ1a <sub>1-62</sub> fused to HopZ1b <sub>59-368</sub>	This study
pUCP20tk::HopZ1a1b-2-HA	pUCP20tk carrying the native promoter of <i>hopZ1a</i> and a chimeric protein with HopZ1a <sub>1-244</sub> fused to HopZ1b <sub>240-368</sub>	This study
pUCP20tk::HopZ1b1a-1-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-58</sub> fused to HopZ1a <sub>63-369</sub>	This study
pUCP20tk::HopZ1b1a-2-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-239</sub> fused to HopZ1a <sub>245-369</sub>	This study
pUCP20tk::HopZ1a1b1a-1-HA	pUCP20tk carrying the native promoter of <i>hopZ1a</i> and a chimeric protein with HopZ1a <sub>1-137</sub> fused to HopZ1b <sub>134-199</sub> and HopZ1a <sub>204-369</sub>	This study
pUCP20tk::HopZ1a1b1a-2-HA	pUCP20tk carrying the native promoter of <i>hopZ1a</i> and a chimeric protein with HopZ1a <sub>1-62</sub> fused to HopZ1b <sub>59-199</sub> and HopZ1a <sub>204-369</sub>	This study
pUCP20tk::HopZ1a1b1a-3-HA	pUCP20tk carrying the native promoter of <i>hopZ1a</i> and a chimeric protein with HopZ1a <sub>1-62</sub> fused to HopZ1b <sub>59-240</sub> and HopZ1a <sub>245-369</sub>	This study
pUCP20tk::HopZ1b1a1b-1-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-133</sub> fused to HopZ1a <sub>138-203</sub> and HopZ1b <sub>200-368</sub>	This study
pUCP20tk::HopZ1b1a1b-2-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-133</sub> fused to HopZ1a <sub>138-244</sub> and HopZ1b <sub>241-368</sub>	This study
pUCP20tk::HopZ1b1a1b-3-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-58</sub> fused to HopZ1a <sub>63-244</sub> and HopZ1b <sub>241-368</sub>	This study
pUCP20tk::HopZ1b1a1b-4-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-58</sub> fused to HopZ1a <sub>63-137</sub> and HopZ1b <sub>134-368</sub>	This study
pUCP20tk::HopZ1b1a1b-5-HA	pUCP20tk carrying the native promoter of <i>hopZ1b</i> and a chimeric protein with HopZ1b <sub>1-58</sub> fused to HopZ1a <sub>63-203</sub> and HopZ1b <sub>200-368</sub>	This study
pUCP20tk::HopZ1a(C141K)-HA	pUCP20tk carrying HopZ1a including its native promoter and with cysteine141 replaced with a lysine	This study
pUCP20tk::HopZ1a(F115A)-HA	pUCP20tk carrying HopZ1a including its native promoter and with phenoalanine115 replaced with an alanine	This study
pUCP20tk::HopZ1a(H185A)-HA	pUCP20tk carrying HopZ1a including its native promoter and with histidine185 replaced with an alanine	This study
pUCP20tk::HopZ1b(K137C)-HA	pUCP20tk carrying HopZ1b including its native promoter and with lysine137 replaced with a cysteine	This study
pUCP20tk::HopZ1b(F111A)-HA	pUCP20tk carrying HopZ1b including its native promoter and with phenoalanine111 replaced with an alanine	This study
pUCP20tk::HopZ1b(H181A)-HA	pUCP20tk carrying HopZ1a including its native promoter and with histidine181 replaced with an alanine	This study
pMDD1	A binary vector with cauliflower mosaic virus 35 s promoter, Kan <sup>R</sup>	Mudgett <i>et al.</i> (2000)
pMDD1::HopZ1a-HA	pMDD1 carrying HopZ1a tagged with HA	Ma <i>et al.</i> (2006)
pMDD1::HopZ1b-HA	pMDD1 carrying HopZ1b tagged with HA	Ma <i>et al.</i> (2006)
pMDD1::HopZ1a1b-1-HA	pMDD1 carrying the chimeric protein with HopZ1a <sub>1-62</sub> fused to HopZ1b <sub>59-368</sub>	This study
pMDD1::HopZ1a1b-2-HA	pMDD1 carrying the chimeric protein with HopZ1a <sub>1-244</sub> fused to HopZ1b <sub>240-368</sub>	This study
pMDD1::HopZ1b1a-1-HA	pMDD1 carrying the chimeric protein with HopZ1b <sub>1-58</sub> fused to HopZ1a <sub>64-369</sub>	This study
pMDD1::HopZ1b1a-2-HA	pMDD1 carrying the chimeric protein with HopZ1b <sub>1-239</sub> fused to HopZ1a <sub>245-369</sub>	This study

program MODELLER (Marti-Renom *et al.*, 2000) was used to generate models for the catalytic core domain of hopZ1a and hopZ1b. The structure of XopD catalytic core (PDB ID: 2OIV) (Chosed *et al.*, 2007) was used as a template in the structural modelling experiment. The three-dimensional model was obtained by optimally satisfying spatial restraints derived from the sequence alignment results.

#### Statistical analysis

Statistical analyses were performed using JMP 8.0 (SAS Institute, Cary, NC, USA).

#### Acknowledgements

We thank Dr Shou-wei Ding for intellectual discussions. USDA Soybean Germplasm Collection and USDA-ARS (Urbana, IL, USA) kindly provided soybean seeds. This work is funded by National Science Foundation (IOS#0847870), University of California – Los Alamos National Laboratory collaborative program on plant diseases, University of California Riverside start-up funds and USDA-AES/CE RSAP grant to W. Ma.

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