Functional mapping of harpin HrpZ of *Pseudomonas syringae* reveals the sites responsible for protein oligomerization, lipid interactions and plant defence induction

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SUMMARY

Harpin HrpZ is one of the most abundant proteins secreted through the pathogenesis-associated type III secretion system of the plant pathogen *Pseudomonas syringae*. HrpZ shows membrane-binding and pore-forming activities *in vitro*, suggesting that it could be targeted to the host cell plasma membrane. We studied the native molecular forms of HrpZ and found that it forms dimers and higher order oligomers. Lipid binding by HrpZ was tested with 15 different membrane lipids, with HrpZ interacting only with phosphatidic acid. Pore formation by HrpZ in artificial lipid vesicles was found to be dependent on the presence of phosphatidic acid. In addition, HrpZ was able to form pores in vesicles prepared from *Arabidopsis thaliana* plasma membrane, providing evidence for the suggested target of HrpZ in the host. To map the functions associated with HrpZ, we constructed a comprehensive series of deletions in the *hrpZ* gene derived from *P. syringae* pv. *phaseolicola*, and studied the mutant proteins. We found that oligomerization is mainly mediated by a region near the C-terminus of the protein, and that the same region is also essential for membrane pore formation. Phosphatidic acid binding seems to be mediated by two regions separate in the primary structure. Tobacco, a nonhost plant, recognizes, as a defence elicitor, a 24-amino-acid HrpZ fragment which resides in the region indispensable for the oligomerization and pore formation functions of HrpZ.

INTRODUCTION

Pseudomonas syringae can infect the above-ground parts of numerous plant species by entering the plant tissues through wounds or natural openings, such as stomata. In its ability to infect plants and to cause plant diseases, *P. syringae* is dependent on the type III secretion system (T3SS). When the bacteria come into contact with a plant, the genes encoding components of the T3SS and the effector proteins dependent on this system are induced (Boureau *et al.*, 2002; Haapalainen *et al.*, 2009; Thwaites *et al.*, 2004). The secretion system helper proteins and effectors are transported from bacteria to host cells through long pilus structures (Brown *et al.*, 2001; Jin and He, 2001; Li *et al.*, 2002; Roine *et al.*, 1997). The main function of the effector proteins delivered into host cells is to suppress host defence (Grant *et al.*, 2006; Guo *et al.*, 2009), and thus promote bacterial survival and multiplication inside the host tissues. In resistant plants, some of the proteins secreted via T3SS can induce a strong defence response, called the hypersensitive response (HR), characterized by local programmed cell death (Mur *et al.*, 2008).

HrpZ, also called harpin, is a major T3SS-dependent protein produced by *P. syringae*. HrpZ shares some characteristics with other harpins from different Gram-negative plant pathogens: it is heat-stable, glycine-rich, lacks cysteine and is secreted to the extracellular space. HrpZ is also partially homologous with HrpN, the main harpin of *Erwinia* and *Pectobacterium* species (Li *et al.*, 2005). Although HrpZ is conserved in all the *P. syringae* pathovars, except in pv. *tabaci* (Taguchi *et al.*, 2001), and it is abundantly secreted under T3SS-inducing conditions, its function in pathogenesis is still conjectural. A simple *hrpZ* knockout mutant had no detectable phenotype in virulence on host plants (Lee *et al.*, 2001b) or in defence induction on nonhost plants (Charkowski *et al.*, 1998). However, the plant defence elicitor activity of HrpZ has been studied by injecting the purified protein

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into plants, and by transgenic expression in nonhost tobacco plants (Tampakaki and Panopoulos, 2000). In an infected host plant, HrpZ secreted by *P. syringae* was localized within the plant cell wall region (Brown *et al.*, 2001).

In vitro, several different activities have been associated with HrpZ protein, which suggests that it could be a multifunctional protein able to interact with several other molecules. HrpZ has been shown to be able to insert into artificial lipid bilayers and to form cation-permeable pores (Lee *et al.*, 2001a,b). HrpZ has also been found to harbour a binding site for peptides with a defined consensus sequence, and has been demonstrated to bind proteins of host origin (Li *et al.*, 2005). Because the discovered peptide-binding activity showed pathovar–host specificity, and HrpZ or the peptide antiserum did not bind to other proteins from *P. syringae*, it was concluded that the consensus peptide probably represents an epitope of a plant protein. Unfortunately, this protein could not be firmly identified. The peptide-binding activity of HrpZ was localized to a region separate from the region acting as an HR elicitor in tobacco plants. Defence induction by HrpZ can also be observed in another nonhost plant: parsley. Engelhardt *et al.* (2009) showed that the defence elicitor activity of HrpZ in parsley cells was separable from the membrane pore-forming function: several HrpZ insertion mutants that were still active in pore formation had lost elicitor activity, whereas a C-terminal fragment was an active elicitor but unable to form pores. These results suggest that the pore formation activity of HrpZ is not the primary cause of plant defence induction, but that some other molecular interactions lead to the incompatible response.

Harpins are likely to have an important function in bacterial virulence, but the nature of this function is still obscure. T3SSs of several animal and human pathogens are assumed to include a protein translocation complex, which is inserted into the host plasma membrane (Blocker *et al.*, 1999; Håkansson *et al.*, 1996). As for T3SS-harboring plant pathogens, *Xanthomonas campestris* secretes a membrane-inserting protein, HrpF, which binds to lipids and has a pore-forming activity. HrpF is essential for interaction with the host plant, and it has been suggested to have a protein translocation function (Büttner *et al.*, 2002). HrpK, a putative translocase secreted by *P. syringae* (Petnicki-Ocwieja *et al.*, 2005), shares some similarity with HrpF. *Ralstonia solanacearum* secretes a harpin-like protein PopA, which is localized in the host plasma membrane in plants, and has been shown to form ion-conducting pores *in vitro* (Racapé *et al.*, 2005). Harpin HrpN of *Erwinia amylovora* has been shown to be required for the translocation of pathogenicity factor DspA/E into plant cells (Bocsanczy *et al.*, 2008), and HrpN also forms pores in lipid vesicles *in vitro* (Engelhardt *et al.*, 2009). The HrpN results raise the question of whether HrpZ could also act in effector protein translocation, because HrpN and HrpZ are partially homologous. A role in translocation has been suggested recently for HrpZ, so

that it would carry out this function together with several other harpin-like proteins (Kvitko *et al.*, 2007). In *P. syringae*, the abundance of different T3SS effectors and helper proteins with overlapping functions renders it more difficult than in *E. amylovora* to confirm the virulence function of each individual protein. Perhaps all the harpin-like proteins of *P. syringae* function to disturb the host cell plasma membrane integrity in order to increase membrane permeability.

Defining the tertiary structure of bacterial harpins has proven to be difficult because of problems caused by protein aggregation. HrpZ of *P. syringae* has been reported previously to form oligomers, up to octamers or decamers (Chen *et al.*, 1998; Tarafdar *et al.*, 2009). In this study, we show that HrpZ can form large oligomeric structures, at least 16-mers, which are not just random aggregates but seem to be regular structures made up of dimers. We also found that HrpZ has a strong affinity to phosphatidic acid (PA), a membrane lipid with a negatively charged headgroup. To map the functions associated with HrpZ, we constructed a series of mutations in the *hrpZ_{pph}* gene, and analysed the resulting truncated proteins. A region near the C-terminus of HrpZ was found to be indispensable for both oligomerization and membrane lipid interactions. Finally, the region of HrpZ eliciting HR in tobacco plants was fine mapped using synthetic peptides.

RESULTS

The large oligomers of HrpZ are made up of at least 16 subunits

In order to determine the supposed oligomeric structure of HrpZ, we studied the native forms of HrpZ_{pph} by several different methods: gel filtration, chemical cross-linking, yeast two-hybrid analysis and native gels with a lipid mimic *N*-perfluoro-octanoic acid (PFO). HrpZ appears in oligomeric form even when solubilized in regular aqueous buffer. The gel filtration profile of purified non-histidine (His)-tagged HrpZ_{pph} (theoretical molecular weight, 35.25 kDa) revealed that it migrated predominantly at a molecular weight of 67 kDa, which is probably a dimer (Fig. 1A). A peak was also detected at 670 kDa, which could be interpreted as a higher order oligomer. Oligomers of HrpZ could be stabilized in solution by chemical cross-linking, and then separated on polyacrylamide gels (Fig. 1B). Compared with the gel filtration profile, there is a relatively small amount of the dimeric form present, which is probably a result of the inefficiency of cross-linking. BS³ is a homobifunctional cross-linker reacting with free amino groups to form amide bonds, and a bridge only forms when the two primary amines are sufficiently close to each other. In the yeast two-hybrid experiment, HrpZ_{pph} was also found to bind to itself, indicating a tendency to form oligomers (Fig. 1C).

Fig. 1 HrpZ forms oligomers. (A) Gel filtration profile of purified HrpZ_{pph} shows that the predominant form of the protein in solution is dimeric, and a minority appears as higher oligomers. Immunoblot analysis of the eluted fractions (small inset) verifies that the UV-detector signal is caused by HrpZ_{pph}, detected with HrpZ antiserum. The arrows indicate the retention volumes of molecular weight standard proteins, and V₀ is the void volume. (B) HrpZ_{pph} oligomers were stabilized in solution with a chemical cross-linker BS³. After incubation at room temperature with or without BS³ (+/-), the HrpZ_{pph} samples were run on 10% sodium dodecylsulphate-polyacrylamide gel, blotted onto nitrocellulose membrane and detected with HrpZ antiserum. (C) HrpZ_{pph} interacts with itself in a yeast two-hybrid test. Yeast transformants were cultured on Complete Supplement Medium (CSM) lacking leucine and tryptophan (-Leu, -Trp). Colonies were picked and cultivated in liquid CSM, and diluted samples of these cell suspensions were then dropped onto CSM plates with or without adenine (+/-). Interaction of the bait and prey proteins results in yeast growth on the medium without adenine and expression of β-galactosidase. Interaction between murine p53 and SV40 large T-antigen serves as a positive control, and AIP7 is a negative (noninteracting) control.

PFO, which solubilizes membrane proteins without disturbing oligomeric structures (Ramjeesingh *et al.*, 1999), was used to analyse the oligomeric forms of HrpZ_{pph} and the homologous protein from *P. syringae* pv. *tomato* (HrpZ_{p_{to}}) in nondenaturing polyacrylamide gels. Protein bands corresponding to monomer, dimer and a large oligomer of HrpZ were invariably detected (Fig. 2). Frequently, there were two dimeric forms with slightly different electrophoretic mobility. Occasionally, a trimer or a tetramer band was also visible, but intermediate forms between the tetramer and the large oligomers could not be detected. Ca²⁺ and Mg²⁺ ions had no effect on the oligomerization pattern of HrpZ when added to the PFO sample buffer at concentrations of 1–10 mM (data not shown). Also, the addition of the calcium chelator ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) did not promote or inhibit HrpZ oligomerization. To determine the size of the high-order oligomers, the relative mobility was measured of 26 lanes on 16 separate native gel blots. The average molecular size of the oligomer of His-tagged HrpZ_{pph} was 716.5 kDa, with a standard

deviation of 52.79, and the average monomer size was 44.52 kDa, which gives 16.09 as the size ratio of the oligomer to the monomer. The glycine-rich region of HrpZ makes it run on polyacrylamide gels higher than expected on the basis of the theoretical molecular weight, which is 37.77 kDa for His-tagged HrpZ_{pph}. The oligomerization pattern of HrpZ_{p_{to}} from *P. syringae* pv. *tomato* DC3000 was identical to that of HrpZ_{pph}, the only difference being that the protein bands appeared higher on the gels because of the larger size of the HrpZ_{p_{to}} protein (Fig. S1, see Supporting Information). Determined from three separate gel blots, the average size ratio of the high-order oligomer and monomer of HrpZ_{p_{to}} was 15.76, approximating to 16. Thus, based on the analysis on the PFO gel system, we can conclude that the HrpZ oligomer probably contains 16 subunits.

HrpZ binds specifically to phosphatidic acid

HrpZ has been shown previously to be able to insert into lipid bilayers and to form pores (Lee *et al.*, 2001b), and it also

Fig. 2 Analysis of HrpZ oligomer size on nondenaturing polyacrylamide gel with *N*-perfluoro-octanoic acid (PFO), a lipid mimic. The relative mobility (R_f) of a protein in the gel is inversely proportional to the logarithm of the molecular weight. The standard proteins (◆) used to determine the size of HrpZ_{pph} native forms (□) were IgM (950 kDa), thyroglobulin (670 and 335 kDa), ferritin (440 kDa), myosin (210 kDa), β-galactosidase (120 kDa), bovine serum albumin (85 kDa), ovalbumin (50 kDa), carbonic anhydrase (33 kDa) and soybean trypsin inhibitor (28.5 kDa). Myosin and the smaller standard proteins were covalently prestained. The inset shows the HrpZ_{pph} profile on a PFO native gel blot, with arrows indicating the monomer and oligomer bands.

showed some binding specificity for phospholipid composition. We tested the lipid-binding specificity of HrpZ_{pph} with commercial PIP Strips membranes displaying different membrane lipids to determine the binding preferences. Surprisingly, we found that, of the 15 different membrane lipids displayed on the strips, phosphatidic acid (PA) was the only one to which HrpZ bound (Fig. 3). To exclude the possibility that the long His-tag could contribute to PA binding, we also tested the binding of non-His-tagged HrpZ_{pph}. The result was again positive, indicating that PA binding is a character of the native HrpZ protein. We also measured the lipid-binding activities of HrpZ by enzyme-linked immunosorbent assay (ELISA) at pH 7.4 and pH 6.0, and confirmed that, in both conditions, HrpZ has a high affinity to PA, but shows no affinity to phosphatidylcholine (PC). At low HrpZ concentrations, binding to PA seems to be noncooperative (Fig. 4). Calcium ions inhibited HrpZ interaction with PA at pH 7.4, but not at pH 6.0 (Fig. S2, see Supporting Information).

Pore formation activity of HrpZ is dependent on membrane lipid composition and HrpZ concentration

Interaction of HrpZ with membranes having different compositions was studied using membrane vesicles loaded with

Fig. 3 HrpZ binds to a membrane lipid: phosphatidic acid. (A) Of the 15 different membrane lipids displayed on a PIP Strips membrane, HrpZ only binds to phosphatidic acid. Lipids presented: 1, lysophosphatidic acid; 2, lysophosphatidylcholine; 3, phosphatidylinositol (PI); 4, PI-3P; 5, PI-4P; 6, PI-5P; 7, phosphatidylethanolamine; 8, phosphatidylcholine; 9, sphingosine 1-phosphate; 10, PI-3,4-P₂; 11, PI-3,5-P₂; 12, PI-4,5-P₂; 13, PI-3,4,5-P₃; 14, phosphatidic acid; 15, phosphatidylserine; 16, blank. HrpZ bound to the lipid strips was detected with HrpZ-specific antiserum.

calcein, a fluorescent compound. The formation of multiple pores by HrpZ causes disintegration of the vesicles, leading to calcein release and fluorescence emission. Little or no pore formation activity was detected when artificial lipid vesicles were prepared from pure PC and phosphatidylethanolamine (PE) or PC and phosphatidylglycerol (PG) without any PA, whereas pores were formed when the vesicles contained 25% PA (Fig. 5A). The rate of calcein release from the PC/PE/PA vesicles was dependent on the concentration of HrpZ in the reaction and, when the concentration was 50 nM or less, no pore formation was detected (Fig. 5B). However, the calcein release rate was lower at 2 μM than at 500 nM protein concentration, suggesting that too high a concentration was inhibitory. When vesicles were prepared from isolated *Arabidopsis thaliana* plasma membranes, the highest pore formation activity was obtained with 20–50 nM HrpZ concentrations (Fig. 5C). At 500 nM concentration, which gave the highest activity rates on the PC/PE/PA vesicles, HrpZ was almost inactive on the plasma membrane vesicles. According to Uemura *et al.* (1995), 46.8 mol.% of total lipids of the plasma membrane from non-acclimated *Arabidopsis* leaves are phospholipids, with PC constituting 35.5 mol.%, PE 38.9 mol.%, PG 9.0 mol.% and PA 6.4 mol.%.

Fig. 4 HrpZ binding to phosphatidic acid (PA) shows no cooperativity at low harpin concentrations. (A) HrpZ binding to immobilized PA, determined by enzyme-linked immunosorbent assay (ELISA), was saturated at HrpZ concentrations higher than 100 µg/L (2.6 nM). Nonspecific binding to wells not coated with lipids (diamonds) was subtracted from the binding to wells coated with PA (open triangles) to obtain PA-specific binding (filled triangles). $n = 4$; the error bars indicate the standard deviation. (B) Hill blot of the PA-specific binding is linear in the middle range, with a slope of 1.08, suggesting that the soluble form of HrpZ binds to PA in a noncooperative manner. The saturation (Y) was assumed to be 100% at an absorbance difference of 2.0.

A series of HrpZ_{Pph} mutants was constructed for functional mapping

To map the different functions associated with HrpZ, we constructed a series of deletion mutants, named ZΔA–J (Fig. 6 and Table 1). A previously created insertion mutant library of the *hrpZ_{Pph}* gene contains random 15-bp insertions with a *NotI* restriction site (Li *et al.*, 2005). When two different insertion mutants, which had the *NotI* site in the same reading frame, were cut and combined, the result was a mutant in which the

Fig. 5 Pore formation activity of HrpZ on lipid vesicles is dependent on protein concentration and membrane composition. (A) HrpZ_{Pph} (200 nM) was applied on vesicles prepared from different combinations of pure lipids (PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol). (B) Effect of HrpZ concentration, from 5 nM to 2 µM, on calcein release from PC/PE/PA vesicles. (C) Effect of HrpZ concentration, from 20 to 500 nM, on calcein release from vesicles prepared from *Arabidopsis thaliana* plasma membrane. The control samples were prepared from *Escherichia coli* transformed with empty vector pJC40, and thus contained no HrpZ.

sequence between the two *NotI* sites was deleted. Because a *NotI* site was restored by the ligation, the final constructs still carry a small insert. Using polymerase chain reaction (PCR), we also constructed a double deletion mutant of HrpZ_{Pph}, Zm, which lacks both the N-terminus and the C-terminal part. Mutated proteins were expressed in *Escherichia coli* with an N-terminal His tag, and then affinity purified. Each one of these truncated versions of HrpZ_{Pph} was tested for oligomerization, pore

Fig. 6 HrpZ_{Pph} mutants were constructed to map the different functions of the harpin. Scale bar represents the whole 345-amino-acid HrpZ_{Pph} sequence. Deletion mutant sequences are shown below the bar as broken lines, in which the gap represents the deleted region. The numbers at the line ends on both sides of the gap indicate the last preserved amino acids, which were joined through a *NotI* linker. The arrow on the scale bar indicates a predicted extended strand region, and the cylinders indicate predicted α -helices. The short lines above the scale bar show the positions of HrpZ_{Pph} fragments P24 and P44.

Table 1 Summary of the characteristics of HrpZ_{Pph} mutants.

Mutant	Amino acids deleted*	Amino acids added by Mu-insertion†	Peptide ZBP1-binding activity‡	Phosphatidic acid (PA)-binding activity§	HR induction in tobacco	Oligomer profile	Membrane pore formation
ZΔA	2–27 (26)	CGR	0.47 (± 0.0335)	0.677	Weak	Altered	+
ZΔB	30–86 (57)	CGRN	0.00 (± 0.0004)	0.603	Enhanced	Altered	+
ZΔC	89–124 (36)	MRPQ	0.00 (± 0.0016)	1.150	Enhanced	Unaltered	+
ZΔD	126–152 (27)	MRPH	0.00 (± 0.0013)	0.074	Enhanced	Unaltered	+
ZΔE	126–143 (18)	MRPQ	0.00 (± 0.0013)	1.358	Enhanced	Unaltered	+
ZΔF	159–194 (36)	AAA	0.00 (± 0.0005)	0.640	Enhanced	Unaltered	+
ZΔG	195–238 (44)	AAA	0.57 (± 0.0626)	0.781	Unaltered	Unaltered	+
ZΔH	241–248 (8)	AAA	0.75 (± 0.0317)	1.089	Unaltered	Defective	+
ZΔI	254–298 (45)	CGRI	0.40 (± 0.0238)	0.117	Negative/weak	Defective	–
ZΔJ	303–335 (33)	RPQ	0.68 (± 0.0621)	1.095	Unaltered	Defective	+
Zm	2–31 and 205–345	—	0.22 (± 0.0445)	0.100	Negative	Defective	–

*Positions of the deleted amino acids in the original non-His-tagged protein sequence; the deletion length is given in parentheses.

†The inserted *NotI* site brought three extra codons in place of the region deleted. In addition, one codon could change.

‡Peptide-binding activity in relation to the full-length HrpZ_{Pph} (=1). $n = 4$; the standard deviation is given in parentheses.

§PA-binding activity in relation to the full-length HrpZ_{Pph} (=1). $n = 6$ –12.

Activities severely impaired by the mutation are marked with shading.

formation, PA binding, peptide binding and nonhost defence induction activities (Table 1).

HrpZ oligomerizes through interactions of the terminal regions

To map the regions of HrpZ that are responsible for oligomerization, we analysed the oligomerization pattern of the deletion mutants on native polyacrylamide gels with PFO. Surprisingly, most of the deletion mutants showed an oligomerization pattern very similar to that of the full-length protein (Fig. 7). The double deletion mutant Zm, in which both the very N-terminal region and the whole C-terminal part of HrpZ were deleted, was unable to form oligomers. Of the single deletion mutants, the mutant ZΔI was the only one that did not show any high-order oligomer band on PFO gels. However, ZΔI still formed dimers, and some-

times also a trimer or a tetramer was detected. For the C-terminal mutant ZΔJ, an indistinct high-order oligomer band was visible only when PFO was omitted from the sample buffer, suggesting that the subunit interaction was weak in this mutant. The high-order oligomer band of mutant ZΔH was also diffuse and sometimes undetectable, indicating instability. Mutants ZΔA and ZΔB showed an altered oligomer pattern, with detectable amounts of intermediate forms between dimers and high-order oligomers. A ladder of intermediate oligomers ascending by double steps became visible on the gel blots, suggesting that the large oligomers were made up of dimers. Higher dimer accumulation was detected with mutants ZΔI, ZΔA and ZΔB. However, this was probably caused by dimer stabilization by S–S bridging between the cysteine residues, which were introduced into these mutants when they were constructed. When dithiothreitol, a reducing agent, was added to the sample buffer, normal levels of

Fig. 7 The C-terminal deletion mutants were defective in oligomer formation. Left: immunoblot of the full-length HrpZ_{Pph} (WT) and HrpZ_{Pph} deletion mutants ZΔA–ZΔJ (A–J), run on 5.5%–8% native polyacrylamide gel electrophoresis (PAGE) with *N*-perfluoro-octanoic acid (PFO). The samples were not heated before loading. Middle: the accumulation of dimers of the mutants ZΔI, ZΔA and ZΔB was reduced when the protein samples were heated at 65 °C in sample buffer containing 100 mM dithiothreitol (final concentration) before loading onto the gel. The full-length protein (WT) was treated similarly to the mutants. Right: immunoblot of mutant Zm run on 6%–12% native PAGE with PFO. Note that this panel is not in the same scale as the two other panels; the molecular weight of Zm is only 21.1 kDa. m, monomer of HrpZ; d, dimer of HrpZ; o, large oligomer of HrpZ.

dimers were detected (Fig. 7, middle panel). The reducing agent did not prevent dimer formation and, for the mutants ZΔA and ZΔB, the intermediate and large oligomers were also detectable. Heating and the addition of reducing agent did not change the oligomerization pattern of the full-length protein, which was expected because HrpZ is heat stable and does not contain cysteine.

A near C-terminal region of HrpZ is essential for both PA binding and pore formation activity

The relative binding affinity of the HrpZ_{Pph} deletion mutants to PA was determined by ELISA using lipid-coated microtitre plates. Mutant ZΔD had lost the specificity of binding to PA, and instead showed a high level of nonspecific hydrophobic adhesion (Fig. 8A). Because the mutant ZΔE with partially overlapping deletion showed high affinity to PA, the amino acid region 144–152 (helix V) seems to be required for PA-binding specificity. Mutants ZΔI and Zm were found to be almost inactive in PA binding, and their nonspecific binding was also at a low level, which demonstrates the importance of the near C-terminal region in hydrophobic interactions. We also tested the membrane pore-forming activity of each HrpZ_{Pph} deletion mutant with two types of lipid vesicle, prepared either from *A. thaliana* plasma membranes or from pure lipids (PC/PE/PA = 3:3:2). Although most of the deletion mutants showed pore formation activity similar to that of the full-length protein on both types of vesicle, two mutants were significantly different (Fig. 8B). First, the mutant ZΔI was consistently inactive in pore formation.

Second, the mutant ZΔF was more active than the full-length protein in terms of calcein release from the PC/PE/PA vesicles at all of the tested time points. Despite the loss of PA-binding specificity in ELISA, the mutant ZΔD was active in pore formation, which suggests that the remaining interaction sites could be adequate for the facilitation of membrane insertion in these conditions. For clarity, the calcein release curves of all of the mutants that performed similarly to the full-length protein are not shown in this figure.

Specific peptide binding is mediated by the N-terminal half of HrpZ

The peptide-binding activity of HrpZ was used in this mutation analysis as a tool to monitor HrpZ native fold and domain structure. When we tested the HrpZ_{Pph} mutants for binding to ZBP1, a HrpZ-binding peptide previously identified by phage display (Li *et al.*, 2005), we found that the deletions covering amino acids 30–194 of HrpZ_{Pph} (ZΔB–F) completely abolished the binding activity (Table 1). This result is in accordance with our earlier results from insertion mutagenesis, locating the peptide-binding domain to the middle part of HrpZ_{Pph} between amino acids 86 and 194 (Li *et al.*, 2005). As a result of the lack of transposon insertions in the *hrpZ_{Pph}* gene between codons 30–61, the role of this region in peptide binding could not be assessed. However, in the highly homologous gene *hrpZ_{Pto}*, several insertions within region 39–144, corresponding to codons 40–121 of *hrpZ_{Pph}*, inhibited peptide binding (C.-M. Li, M. Haapalainen and S. Taira, unpublished results).

Fig. 8 Phosphatidic acid (PA) binding and pore formation activities of the HrpZ_{Pph} mutants. (A) HrpZ_{Pph} mutants Z_{AD}, Z_{AI} and Z_m were defective in PA binding in enzyme-linked immunosorbent assay (ELISA). Black bars represent PA-specific binding at pH 6.0, with protein concentration of 2.6 mM. Control, no HrpZ added. $n = 6-30$; error bars indicate the standard deviation. Results for HrpZ_{Pto} are shown for comparison. (B) Mutant Z_{AI} was unable to form pores in lipid vesicles. The pore formation activities of the full-length HrpZ_{Pph} (wt) and the mutants Z_{AD}–J (D, I, J and F shown) were determined as the percentages of calcein released from vesicles prepared from pure lipids (phosphatidylcholine/phosphatidylethanolamine/PA = 3:3:2). Protein concentration was 200 nM; $n = 3$; the error bars indicate the standard deviation. The negative control is a protein preparation of *Escherichia coli* carrying the empty expression vector pJC40.

A short fragment of HrpZ is recognized as an elicitor of HR in tobacco plants

The previously constructed HrpZ_{Pph} insertion mutants, carrying five amino acid insertions at random locations, all still retained HR elicitor activity on tobacco (C.-M. Li, unpublished results). Therefore, we tested the series of deletion mutants for HR induction in tobacco, and found that all but one of the single deletion mutants retained the activity. The mutant Z_{AI} was invariably

negative in the HR induction tests (Fig. 9). In addition, with mutant Z_{AA}, the tobacco response was variable: sometimes weakly positive and sometimes negative. Mutants Z_{AB}, Z_{AC}, Z_{AD}, Z_{AE} and Z_{AF} induced a stronger response than the full-length protein, which often induced a partial collapse of the infiltrated area. The double deletion mutant Z_m did not induce HR, which was expected, as this mutant lacks the whole C-terminal part of the harpin, previously shown to be responsible for HR induction (Lee *et al.*, 2001a). However, Z_m still induced slight chlorosis of the infiltrated area.

To fine map the location of the HR elicitor activity, synthetic peptides representing putatively α -helical fragments of the HrpZ_{Pph} sequence were prepared and tested on tobacco leaves. Peptides P44 (amino acids 269–312) and P24 (amino acids 290–313) induced cell death in the whole infiltrated area, whereas the other peptides representing different regions of the protein did not induce HR (Table 2). Because the peptides P24 and P44 were consistently inactive in pore formation assay on both PC/PE/PA and plant plasma membrane vesicles (data not shown), we can conclude that HR induction in tobacco by HrpZ does not require membrane pore formation. At first sight, the result that P24 induces HR on tobacco may seem contradictory with the result that the mutant Z_{AI} (amino acids 303–335 deleted) is fully active as an HR elicitor. P24 is a putatively α -helical amino acid motif, of which there are three slightly variable repeats in the C-terminal region of HrpZ (Table 3). By chance, when constructing the mutant Z_{AI}, an in-frame chimera of repeats II and III was created which apparently resulted in the restoration of HR elicitor activity. This observation, together with sequence similarity to other harpin-derived HR elicitors, supports the concept that there is a consensus sequence for HR elicitation in tobacco by the harpins (Table 3). Because, on parsley cells, two of the HrpZ_{Pph} mutants, Z_{AB} and Z_{AI}, showed reduced phytoalexin inducer activity, and the peptides P24 and P44 showed no inducer activity (data not shown), the other nonhost plants may recognize some other fragments of HrpZ as defence elicitors.

DISCUSSION

We studied the molecular interactions and degree of oligomerization of HrpZ harpin from *P. syringae*, and mapped the regions involved in each interaction by the functional analysis of a series of mutants. In this way, we identified the structural requirements for the different activities, and obtained a function-based structural model. However, it is difficult to reconcile the several different functions of HrpZ into one mode of action; all the functions do not seem to be directly linked to each other, and hence it is futile to forcibly link them together into one complete model. The picture of HrpZ that is emerging is that it may be characterized as a true multifunctional protein with several alternating tertiary and quaternary structures.

Fig. 9 A 24-amino-acid fragment of HrpZ is sufficient to elicit a full hypersensitive response (HR) in tobacco. Full-length HrpZ_{Pph} and mutants ZΔA–J and Zm, and also synthetic peptides representing fragments of HrpZ_{Pph}, were tested for HR induction in tobacco (*Nicotiana tabacum* cv. Samsun). Samples of purified proteins and peptides were prepared in 5 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, pH 5.5, to 0.5 mg/mL concentration, and infiltrated into tobacco leaves. At least three replicates of each sample were applied in each experiment, and the plant response was observed for 72 h.

Table 2 Synthetic HrpZ_{Pph}-derived peptides tested for hypersensitive response (HR) induction.

HrpZ fragment	Amino acid sequence	HR
33–51 (helix I)	SSRALQEVIAQLAQELTHN	–
59–72 (helix II)	PLGKLLGKAMAASG	–
77–94 (helix III)	GLEDIKAALDTLIEKLG	–
108–130 (helix IV)	GQHDLMTQVLNGLAKSMLNDLLT	–
143–154 (helix V)	MPMLKKIAEFMD	–
180–205 (helix VI)	GDETAQFRSALDIIGQQLGSSQNAAG	–
249–269 (helix VII)	GDVGQLIGELIDRGLQSVLAG	–
269–312 (random coil and helix VIII) P44	GGGLGTPVSTANTALVPGGEQPNQDLGQLLGGLLQKGLEATLQD	+
290–313 (helix VIII) P24	PNQDLGQLLGGLLQKGLEATLQDA	+
319–339 (helix IX)	GVQSSAAQVALLVNMLLQST	–

Table 3 Harpin-derived amino acid motif recognized as a hypersensitive response (HR) elicitor by tobacco plants.

Harpin, codon positions	Amino acid sequence	HR on tobacco
HrpZ- <i>Pph</i> , I (250–264)	DVGVQLIGELIDRGLQ	Negative
HrpZ- <i>Pph</i> , II (293–307)	DLGQLLGGLLQKGLE	Positive
HrpZ- <i>Pph</i> , III (323–337)	SAAQVALLVNMLLQ	Negative
HrpZ- <i>Pph</i> , ZΔJ	DLGQLLGGLLRPQLQ	Positive
HrpZ- <i>Pph</i> insertion mutant G300	DLGQLLGGGRIGGLLQKGLE	Positive
HrpZ- <i>Pph</i> insertion mutant G302	DLGQLLGGLLRPHLQKGLE	Positive
HrpZ- <i>Pph</i> insertion mutant K304	DLGQLLGGLLQCGRMQKGLE	Positive
XopA- <i>Xcv</i> (33–55)	QLDQLLTQFIIFSMLL	Negative*
Hpa1- <i>Xoo</i> (32–54)	QLDQLLCOILI SALLQ	Positive (weak)*
HpaG- <i>Xag</i> (31–53)	QLDQLLTQLIMALLQ	Positive (strong)*
HrpN- <i>Ep</i> (102–113)	GLGGLLGGDLGST	Positive†
Elicitor consensus with variations	qLdQLLtqLIxxxLQ d g gg L E	

Note that the sequence of the mutant ZΔJ is a chimera of the repeats II and III of HrpZ_{Pph}. Similar motifs found from other bacterial harpins and tested for HR induction are shown for comparison. *Ep*, *Erwinia pyrifoliae*; *Pph*, *Pseudomonas syringae* pv. *phaseolicola*; *Xag*, *Xanthomonas axonopodis* pv. *glycines*; *Xcv*, *X. campestris* pv. *vesicatoria*; *Xoo*, *X. oryzae* pv. *oryzae*. Conserved amino acids are framed, and divergent amino acids in the HR negative variants are shaded.

*Results from Kim *et al.* (2004).

†Result from Shrestha *et al.* (2008).

Fig. 10 Functional domains of HrpZ_{Pph}. All the different functions characterized and mapped in this study are summarized in this two-domain structural model. The numbers refer to amino acids, 345 in total. Note that the C-terminal domain alone is not capable of forming pores in lipid membranes, and other parts of the protein are also needed to carry out this function (Engelhardt *et al.*, 2009). HR, hypersensitive response; PA, phosphatidic acid.

We found that, in solution, HrpZ is mainly a dimer, and the dimers join to form a large oligomeric structure with an estimated molecular size of 670 kDa. The two-top dimer peak in the gel filtration profile and the two separate dimer bands in the PFO gels suggest that there are two dimeric forms differing in conformation and revealed by different mobility. The oligomerization pattern of HrpZ, showing dimers and large oligomers and lacking intermediates, is unique, and suggests that the association of dimers to higher order oligomers is rapid and cooperative. Our results are in good agreement with the dynamic light scattering data of HrpZ_{Pssr} recently published by Tarafdar *et al.* (2009), showing that HrpZ forms both dimers and higher order oligomers. However, the tertiary structural model proposed by Tarafdar *et al.* (2009) for HrpZ is not consistent with the secondary structural predictions or with our results on functional mapping. Secondary structural predictions consistently give nine α -helices for HrpZ, and these structures are conserved in the HrpZ variants from different *P. syringae* pathovars. However, for the HrpZ tertiary structure, the modelling programs give several different suggestions with evenly low probability scores, because there are no experimental data available on the three-dimensional structure of any harpin-like protein on which the model could be based. Moreover, putative leucine zipper motifs from different harpins were aligned to show that they would have identical functions in protein oligomerization. However, when we analysed HrpN, a harpin originating from *Pectobacterium carotovorum*, on PFO gels, no dimers or higher order oligomers were detected (data not shown). Instead, PopA, a harpin-like protein of *Ralstonia solanacearum*, has been found previously to form oligomers (Racapé *et al.*, 2005) but, different from HrpZ, the trimeric form was abundant.

In general, the HrpZ deletion mutants retained the protein functions surprisingly well. HrpZ is able to renature after boiling and fold on its own, and even this ability was still retained by the mutants. In addition, all the mutants with a single deletion were still able to form dimers, suggesting that the dimeric bond is

strong and formed by large surface areas. Thus, the structure of HrpZ seems to be very flexible, allowing large modifications whilst retaining at least partial functionality. This may be an indication of a modular structure: if a protein consists of separately folding domains, mutation in one domain does not inactivate another domain. At the subdomain level, if there are similar structural motifs in succession, one motif could replace another which has been removed, and restore functionality. Some of the nine α -helices of HrpZ could function as subdomain modules [for α -helix stability, see the circular dichroism spectra in Fig. S3 (Supporting Information)]. However, deletion of amino acids 254–298 (ZΔI) had more dramatic consequences than the other mutations, clearly indicating that this region is structurally important, probably forming the core of the C-terminal domain. Because the mutant ZΔI lost both the ability to form large oligomers and the ability to form pores in membrane vesicles, these two activities are likely to be linked. However, a partial impairment in oligomer formation (or oligomer instability) of the other C-terminal mutants ZΔH and ZΔJ did not prevent pore formation, suggesting that the HrpZ structure may be stabilized by the membrane lipid bilayer, or that there is a mild effect which is undetectable by the calcein release assay. Oligomerization did not seem to be affected by mutations within the region 89–238 of HrpZ_{Pph}, harbouring the previously characterized peptide-binding site. Moreover, the mutant ZΔI still retained peptide-binding activity, although reduced, suggesting that the three-dimensional structure of the peptide-binding region remained almost intact despite the destruction of the C-terminal functions. These results support a structural model with two functional domains (Fig. 10). Deletion of the N-terminal 27 amino acids altered several interactions, and thus the N-terminus is likely to be an important part of the native protein fold. For one of the mutants, ZΔG, no phenotype was found, except for the enhanced electrophoretic mobility on polyacrylamide gels (Fig. 7). The region deleted in this mutant is rich in glycine and serine—of the 44 amino acids, 10 are glycine and seven are serine—and the

computed secondary structural prediction is almost entirely a random coil. This unstructured region may act as a flexible linker between the N-terminal and C-terminal functional domains of HrpZ, allowing the domains to move and repose in relation to each other to mediate different molecular interactions.

As HrpZ has been found previously to bind to lipids and to insert into membranes, it was expected that it would show some specific affinity to membrane lipids. The surprise was that, in the lipid strip assay, HrpZ only showed affinity to PA. The observation that HrpZ did not form pores in artificial lipid vesicles free of PA strengthens the results of the lipid-binding assays. These new pore formation results might seem to be contradictory with some previous results (Lee *et al.*, 2001b); however, a probable explanation for the difference is that the former lipid preparations may not have been completely free of PA, which is formed as a degradation product of the other phospholipids. Moreover, the previous electrophysiological assays are more sensitive and may supersede the lower affinity of HrpZ for other lipid compositions. In a follow-up study (Lee *et al.*, 2001a), a nonproteinaceous nature of HrpZ-binding sites in tobacco microsomal membranes was suggested and, consistently, the HrpZ-binding site in parsley microsomal membranes was found to be heat and protease resistant (Engelhardt *et al.*, 2009). Consistent with this, it seems probable that PA (or another endogenous lipid with similar properties) is the primary binding site of HrpZ in plant membranes. We also found that HrpZ was active in forming pores in vesicles prepared from *A. thaliana* plasma membrane. PA is a natural minor constituent of plasma membranes and, in *Arabidopsis* leaves, PA constitutes 3.0% of total plasma membrane lipids (Uemura *et al.*, 1995). The plasma membranes also contain many proteins, approximately equal to the amount of lipids, and thus plasma membrane vesicles would contain far fewer docking sites for HrpZ than would artificial lipid vesicles containing 25% PA. This difference in binding site frequency could explain the 10-fold difference in the optimal HrpZ concentrations in pore formation assays. The reason why excessive amounts of HrpZ in the reaction mixture decrease pore formation is not clear. It may be that the excessive free protein aggregates and is no longer active in pore formation.

PA seems to be required by HrpZ for membrane insertion, perhaps to create suitable membrane topography. As a result of the conical shape of the PA molecule, a membrane region rich in PA has negative curvature, which is supposed to enhance protein binding to these regions (Kooijman *et al.*, 2007). The characterized PA-binding sites of different proteins do not seem to be homologous to each other. However, an abundance of charged and aromatic residues is common to all of the known binding site sequences (Stace and Ktistakis, 2006). Positively charged lysine and arginine residues bind to the negatively charged headgroup of PA, and large hydrophobic residues, such as phenylalanine and tryptophan, are needed for binding to acyl chains

(Kooijman *et al.*, 2007). The HrpZ sequence shows no homology to the published PA-binding sites, which are from endogenous eukaryotic proteins. We found that mutations in two regions of HrpZ_{pph}, amino acids 144–152 and 254–298, resulted in a loss of PA-binding activity in ELISA. In the hydrophilic middle region of HrpZ_{pph}, there are several lysine residues which could bind to the negatively charged headgroup of PA. The previous result that HrpZ binding to tobacco microsomes was inhibited at high pH (Lee *et al.*, 2001a) refers to ionic interaction and the involvement of lysine residues, because when the lysines deprotonate they can no longer bind to the phosphate group. The more hydrophobic terminal regions of HrpZ could bind to the lipid fatty acid moiety. In addition to being a structural component of the membranes, PA is also an essential intermediate in lipid biosynthesis and an important signalling molecule in cellular stress (reviewed by Testerink and Munnik, 2005). Growing evidence suggests that PA has an important role as a second messenger in the plant defence response against pathogens (Arisz *et al.*, 2009). Thus, an intriguing question is whether the PA-binding activity of HrpZ might also have a role in interfering with the defence signalling function of PA.

Another harpin-like protein, PopA of *R. solanacearum*, has been reported previously to form ion-conducting pores and to require Ca²⁺ for both lipid binding and oligomer formation (Racapé *et al.*, 2005). In contrast, we found that calcium was not required for HrpZ oligomerization, and that the addition of Ca²⁺ had no effect on HrpZ PA-binding activity at pH 6. At pH 7.4, calcium inhibited HrpZ binding to PA, which was probably caused by Ca²⁺ binding to the di-anionic form of PA (Medvedev *et al.*, 2006). PopA was reported to be unselective for the phospholipid composition of lipid bilayers, whereas HrpZ favours negatively charged phospholipids (Lee *et al.*, 2001b; and this study). Thus, although PopA and HrpZ might serve an analogous function in bacterial infection, they seem to employ different mechanisms in their membrane interaction.

In some plants, HrpZ can induce HR, leading to local cell death (He *et al.*, 1993; Preston *et al.*, 1995). HR elicitor activity has been localized previously in the C-terminal part of HrpZ (He *et al.*, 1993; Lee *et al.*, 2001a), or in both the C-terminal and N-terminal parts (Alfano *et al.*, 1996). A C-terminal 148-amino-acid-long fragment of HrpZ_{pss} was found to be four times more potent as an HR elicitor than the full-length protein (He *et al.*, 1993) and, consistently, several of our HrpZ_{pph} deletion mutants showed enhanced HR elicitor activity. Using synthetic peptides representing short fragments of HrpZ, the region eliciting HR in tobacco could be confined to 24 amino acids. This fragment covers the eighth putative α -helix of HrpZ, which is highly conserved in the HrpZ proteins of different *P. syringae* pathovars. Because this region, P24, overlaps with the region deleted in the mutant Δ I, the HR-negative phenotype of Δ I is probably caused by the removal of a part of P24. In the α -helical confor-

mation, the hydrophobic leucine residues would be on one side and the more hydrophilic residues on the other, resulting in an amphipathic structure.

P24 shares similarity with a 23-amino-acid-long HR-active peptide derived from HpaG harpin of *Xanthomonas axonopodis* (Kim *et al.*, 2004) (Table 3). This fragment of HpaG is α -helical and very important for correct protein folding (Wang *et al.*, 2007). A 12-amino-acid-long fragment of the N-terminal half of *Erwinia pyrifoliae* HrpN has been shown to enhance HR elicitation in tobacco, together with two other fragments which both contain a predicted α -helix structure (Shrestha *et al.*, 2008). An interesting question is whether the structural motif shared by several harpins and recognized by some plants serves a similar function in the different harpins, e.g. in protein folding or membrane insertion. Functional mapping of HrpN of *E. amylovora* (HrpN_{Ea}) revealed that the virulence function on host plants and the avirulence activity on nonhost tobacco were not structurally separable (Sinn *et al.*, 2008). Both of these activities, and also the secretion signal, were located in the highly conserved C-terminal half, which is homologous to the middle region of HrpZ (amino acids 125–193 in HrpZ_{Pph}). Instead, the cell-free HR elicitor activity was located in the N-terminal part of HrpN_{Ea} and HrpN_{Ep} (Shrestha *et al.*, 2008; Sinn *et al.*, 2008). Sinn *et al.* (2008) hypothesized that tobacco plants could recognize HrpN_{Ea} by two different mechanisms, depending on whether the harpin is delivered by bacteria or applied as a cell-free preparation. We discovered that, in HrpZ, the fragment recognized as an HR elicitor resides in the region indispensable for protein oligomerization and membrane insertion activities. Thus, it may be impossible to mutate this region unrecognizable without destroying protein functionality. Supporting this idea, the tobacco pathogen *P. syringae* pv. *tabaci* has a truncated *hrpZ* gene and does not produce HrpZ protein (Taguchi *et al.*, 2001), suggesting that it might have evaded recognition and defence induction in tobacco by abandoning HrpZ. It is likely that tobacco specifically recognizes the leucine-rich α -helical structures of bacterial harpins as a pathogen-associated molecular pattern.

EXPERIMENTAL PROCEDURES

Construction of deletion mutants

To map the HrpZ functions, we made 10 successive deletions, named Δ 1–10, in the *hrpZ*_{Pph} gene derived from *P. syringae* pv. *phaseolicola* (Table 1). In addition, we constructed a double deletion mutant Zm, in which both the N-terminal and C-terminal regions were deleted. For the construction of mutants Δ 1–10, we utilized the previously created insertion mutant library of the *hrpZ*_{Pph} gene (Li *et al.*, 2005). Two insertion mutants carrying an introduced *NotI* site at different locations were cut with this enzyme, and the N-terminus encoding part of the

upstream mutant and the C-terminus encoding part of the downstream mutant were ligated. Thus, the region between the two *NotI* sites was deleted, with the addition of three codons originating from the restored restriction site. All the *hrpZ* gene constructs were built in the pJC40 vector, so that the proteins were expressed with an N-terminal polyhistidine tag (MGHHHHHHH-HHHSSGHIEGRH). To produce native 345-amino-acid-long HrpZ_{Pph} without a His-tag, the *hrpZ* gene was cloned into the pJC20 expression vector (Clos and Brandau, 1994). The gene variant encoding mutant Zm, which only comprises amino acids 32–204 of HrpZ_{Pph}, was created by PCR. The forward primer sequence was AACCGGGCATATGACATCCAGCCGGGCG and the reverse primer sequence was CAGAAGCTTCAGGCTGCATTCTGT TGAC. The PCR product was cut with *NdeI* and *HindIII*, and cloned into the pJC40 expression vector.

Harpin expression and purification

HrpZ of *P. syringae* pv. *phaseolicola* race 6 (HrpZ_{Pph}), both the full-length protein and the deletion mutants, was expressed by isopropyl- β -D-thiogalactopyranoside (IPTG) induction in *E. coli* strain BL21 (DE3) pLysS, as described previously (Lee *et al.*, 2001a, b; Li *et al.*, 2005). After removing most of the *E. coli* proteins by heat treatment, the His-tagged HrpZ_{Pph} variants were purified by affinity chromatography, using nickel-nitrilotriacetate (Ni-NTA) agarose (Qiagen, Hilden, Germany) under native conditions. Full-length HrpZ of *P. syringae* pv. *tomato* DC3000 (HrpZ_{Pto}) was similarly produced and purified. The purified HrpZ variants were dialysed twice against 5 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer, pH 5.5, and stored at -70°C . Protein concentrations were determined by densitometry from samples run on sodium dodecylsulphate-polyacrylamide gels, using bovine serum albumin as the quantitative standard. When necessary, the His-tag was removed by treatment with factor Xa protease (New England Biolabs, Ipswich, MA, USA), which cleaves after the arginine residue of the sequence Ile-Glu/Asp-Gly-Arg.

Synthetic peptides

Peptides P44 and P24 were synthesized at the Leibniz Institute of Plant Biochemistry as described previously (Li *et al.*, 2005), and the other peptides representing putatively α -helical fragments of HrpZ_{Pph} were synthesized with better than 95% purity by Peptide Protein Research Ltd. (Fareham, Hampshire, UK). HrpZ secondary structural predictions were made using the Jpred3 program (University of Dundee), available at the ExPASy Proteomics Server net pages. The peptide sequences are presented in Table 2.

Analysis of HrpZ oligomers

Initially, the native molecular size of HrpZ was studied by gel filtration chromatography. Affinity-purified HrpZ_{Pph} was loaded

onto a Superdex 200 (10/300 GL) gel filtration column (Äkta Explorer; Amersham Biosciences, GE Healthcare Biosciences, Uppsala, Sweden) at a concentration of 1 mg/mL in 5 mM MES buffer, pH 5.5, with 150 mM NaCl, and eluted with the same buffer. Protein was detected with a UV detector at two different wavelengths: 280 nm is specific for aromatic residues and, because there are only a few of them in HrpZ, absorption at 214 nm for peptide bonds was also measured to obtain a stronger signal. Eluted fractions were collected and HrpZ was identified by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting.

Second, a chemical cross-linker BS³ (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) was used to stabilize HrpZ_{pph} oligomers in solution. HrpZ_{pph} (0.2 µg/µL) was incubated with 3 mM BS³ cross-linker in 5 mM MES buffer, pH 5.5, for 1 h at room temperature. The reaction was stopped by the addition of denaturing SDS-PAGE loading buffer. Protein samples were run by 10% SDS-PAGE and then blotted onto nitrocellulose membrane. HrpZ was detected with specific rabbit antiserum.

Third, binding of HrpZ to itself was studied by yeast two-hybrid test. Both the bait protein-encoding vector pGBKT7 and the prey protein-encoding vector pGADT7 were transformed into the yeast strain pJ69-4A. Initially, yeast transformants were plated onto Complete Supplement Medium (CSM, Formedium, Norwich, UK) lacking leucine and tryptophan (–Leu, –Trp). Colonies were picked and cultivated in liquid CSM (–Leu, –Trp). Subsequently, a dilution series (10⁰–10^{–3}) was prepared of each yeast cell suspension [optical density at 600 nm (OD₆₀₀) = 0.3], and a 7.5-µL sample of each dilution was applied to CSM agar with adenine (–Leu, –Trp, +adenine) and CSM without adenine (–Leu, –Trp, –adenine). The plates were incubated for at least 3 days prior to photographing. Positive interaction between the expressed proteins resulted in yeast growth and the activation of β-galactosidase whether or not adenine was present. The interaction between murine p53 and SV40 large T-antigen served as the positive control, and AIP7 protein from *Arabidopsis* (C. Grefen and K. Harter, University of Tübingen, unpublished) served as a noninteracting negative control.

Fourth, in order to compare the oligomerization patterns of the HrpZ_{pph} mutants, electrophoresis in nondenaturing polyacrylamide gels with PFO (Apollo Scientific, Stockport, England) was performed. Protein samples were run on native 5.5%–8.0% gradient gels in Tris-glycine buffer with 0.5% PFO, which solubilizes membrane proteins without disturbing the oligomeric structures (Ramjeesingh *et al.*, 1999). The sample loading buffer and gel running buffer were prepared according to Ramjeesingh *et al.* (1999). On the basis of protein concentration measurements, the amount of each protein sample was adjusted to 5–6 µg per well. After running the gel at 4–8 °C, the proteins were blotted onto a polyvinylidene difluoride membrane. The molecular weight standard proteins were blue stained, and HrpZ variants were

detected by HrpZ-specific rabbit antiserum and monoclonal anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA), followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) colour reaction. The relative mobility (*R_i*) of each protein band was determined, and the HrpZ oligomer/monomer ratios were calculated.

Lipid-binding assays

The lipid-binding preference of HrpZ was first tested on PIP Strips membranes (Molecular Probes, Invitrogen, San Diego, CA, USA). The strips carrying samples of 15 different lipids plus a negative control were pre-incubated in 10 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 0.05% (v/v) Tween 20 and 3% bovine serum albumin (fraction V, Roche, Basel, Switzerland) for 1 h. After 2 h of incubation with HrpZ, 1 µg/mL, the excess protein was washed away with the same buffer. Protein bound to the lipid blots was detected with HrpZ antiserum and anti-rabbit alkaline phosphatase conjugate, followed by the addition of the chromogenic substrate NBT/BCIP (Promega, Madison, WI, USA). All the incubations and washing steps were performed at room temperature (22 ± 1 °C).

To compare the PA-binding affinities of the HrpZ_{pph} mutants, ELISA was used. Microtitre plate wells were coated with 100 µL of dipalmitoyl phosphatidic acid (Sigma), first dissolved in a mixture of chloroform and methanol (2:1), 2 mg/mL, and then diluted with methanol to 20 µg/mL. The blank wells were 'coated' with 100 µL of methanol only. The solvent was evaporated to dryness overnight at room temperature. The coated plates were washed once with MES-buffered saline (MES-S: 10 mM MES buffer, pH 6.0, 150 mM NaCl), and blocked with 5% nonfat dry milk (NFDm) in MES-S for 2 h. After washing with 0.5% NFDm/MES-S, 50 µL of HrpZ variants diluted to 2.6 nM in the same buffer were added, and incubated with gentle rocking for 3 h. The plates were washed three times with 0.5% NFDm/MES-S before incubation with the HrpZ antiserum for 60 min, and then washed four times with 0.05% Tween/MES-S. After 30 min of incubation with alkaline phosphatase-conjugated anti-rabbit antibodies, the plates were again washed four times with 0.05% Tween/MES-S. A chromogenic substrate for alkaline phosphatase, 4-nitrophenyl phosphate (Sigma), 1 mg/mL in diethanolamine MgCl₂ buffer, was added, and the absorbance at 405 nm was measured after 60 min of incubation at 37 °C. All the steps preceding the final colour reaction were performed at room temperature.

Pore formation assay

Lipid vesicles loaded with calcein, a fluorescent dye, were prepared in a similar manner to that described previously for plasma membrane vesicles (Ottmann *et al.*, 2009). For this work, vesicles

were prepared of both *A. thaliana* plasma membrane and of mixtures of pure lipids. PC, PE, PG and PA were used to construct three types of pure lipid vesicle in ratios of PC/PE/PA 3:3:2, PC/PE 8:2 and PC/PG 98:2. The vesicles were suspended in 20 mM MES buffer, pH 5.8, with 140 mM NaCl. The protein samples to be tested were mixed with the vesicle suspension in microtitre plate wells to final protein concentrations of 5–2000 nM in a reaction volume of 200 µL. The fluorescence emission was monitored for 15 min (excitation at 485 nm, emission at 520 nm). The maximal fluorescence (100%) was obtained by adding Triton X-100 to 0.5% (v/v), which completely disrupts the lipid vesicles. A protein preparation of *E. coli* carrying the empty expression vector pJC40 served as the negative control sample.

Peptide-binding assay

The binding of phage-displayed peptides to HrpZ was determined by ELISA in a similar manner to that described previously (Li *et al.*, 2005). Briefly, the microtitre plate wells were coated with HrpZ, incubated first with the recombinant phages, then with M13-specific antibody (GE Healthcare, Little Chalfont, Buckinghamshire, England) and finally with polyclonal anti-mouse antibodies conjugated with alkaline phosphatase (Dako, Copenhagen, Denmark). The absorbance at 405 nm was measured 60 min after the addition of the 4-nitrophenylphosphate substrate solution.

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SUPPORTING INFORMATION

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

Fig. S1 HrpZ forms oligomers. HrpZ proteins were produced with an N-terminal His-tag (see Experimental procedures). Protein samples were run on nondenaturing polyacrylamide gel with *N*-perfluoro-octanoic acid (PFO) (a lipid mimic) and blotted onto polyvinylidene difluoride (PVDF) membrane. HrpZ bands were detected with specific antiserum and colour reaction. St, prestained molecular weight standards (myosin, 210 kDa; β -galactosidase, 120 kDa; bovine serum albumin, 85 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 28.5 kDa); Pph, HrpZ from *Pseudomonas syringae* pv. *phaseolicola*; Pto, HrpZ from *P. syringae* pv. *tomato* (DC3000).

Fig. S2 HrpZ binding to dipalmitoyl phosphatidic acid (PA) was not affected by 2 mM calcium ion or by the calcium chelator ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) at pH 6.0. At pH 7.4, calcium inhibited the interaction between HrpZ and PA, probably by binding to the di-anionic form of PA. HrpZ_{Pph} binding to PA was determined by enzyme-linked immunosorbent assay (ELISA), as the difference between PA-coated and noncoated wells, blocked with bovine serum albumin (BSA). *n* = 6; error bars indicate standard deviation.

Fig. S3 Circular dichroism spectra of HrpZ_{p_{ph}} indicate that the protein is mostly α -helical. HrpZ_{p_{ph}} concentration was 0.1 mg/mL (2.6 mM) in 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5. Gradual addition of n-octyl-beta-D-glucopyranoside (OGP) to the protein solution caused no change in the spectra.

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