Harpin of *Pseudomonas syringae* pv. *phaseolicola* Harbors a Protein Binding Site

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Submitted 8 March 2004. Accepted 7 September 2004.

Harpin HrpZ of plant-pathogenic bacterium *Pseudomonas syringae* elicits a hypersensitive response (HR) in some nonhost plants, but its function in the pathogenesis process is still obscure. HrpZ-interacting proteins were identified by screening a phage-display library of random peptides. HrpZ of the bean pathogen *P. syringae* pv. *phaseolicola* (HrpZ_{Pph}) shows affinity to peptides with a consensus amino acid motif W(L)ARWLL(G/L). To localize the peptide-binding site, the *hrpZ*_{Pph} gene was mutagenized with randomly placed 15-bp insertions, and the mutant proteins were screened for the peptide-binding ability. Mutations that inhibited peptide-binding localized to the central region of *hrpZ*_{Pph}, which is separate from the previously determined HR-inducing region. Antiserum raised against one of the *hrpZ*_{Pph}-binding peptides recognized small proteins in bean, tomato, parsley, and *Arabidopsis thaliana* but none in tobacco. On native protein blots, *hrpZ*_{Pph} bound to a bean protein with similar pI as the protein recognized by the peptide antiserum. The result suggests a protein–protein interaction between the harpin and a host plant protein, possibly involved in the bacterial pathogenesis.

Additional keywords: functional domains, HrpN, type III secretion system.

Pseudomonas syringae can infect the aboveground parts of numerous plant species by entering the plant tissues through wounds or natural openings, such as stomata. When bacteria get inside the plant, bacterial genes encoding the components of the type III secretion system (TTSS) are induced (Boureau et al. 2002). A variety of co-regulated genes encode effector proteins that are secreted through this system and transported from the bacterial cell into the plant cell cytoplasm through a long pilus structure (Brown et al. 2001; Jin and He 2001; Li et al. 2002). The presumed function of the proteins delivered to the host cell via TTSS is to inhibit or suppress the host defense and thus promote bacterial survival and multiplication inside the host tissue (Galán and Collmer 1999). Accordingly, TTSS is essential for the ability of *P. syringae* to infect plants and to cause plant diseases (Roine et al. 1997).

One of the proteins secreted via TTSS is HrpZ, also referred to as harpin, which is not delivered into the host cytoplasm but

into the intercellular space. The function of HrpZ in the bacterial infection process is not known. At high concentrations, HrpZ induces a local programmed cell death called hypersensitive response (HR) in some nonhost plants (He et al. 1993; Preston et al. 1995). Tobacco (*Nicotiana tabacum*) has been favored as a test plant for HR induction by harpins, because it gives a strong and repeatable response. Interestingly, *P. syringae* pv. *tabaci*, which causes wild fire disease of tobacco, does not produce HrpZ but only carries a truncated, nonfunctional copy of the *hrpZ* gene (Taguchi et al. 2001). Thus, in tobacco, expression of HrpZ might be deleterious rather than beneficial for the bacterial pathogen. Each *P. syringae* pathovar seems to have developed an individual arsenal of proteins secreted through the TTSS (Charity et al. 2003); therefore, HrpZ also may have evolved to interact specifically with the plants included in the pathovar host range.

Results with in planta expression of HrpZ indicate an extra-cellular function: HrpZ elicits an HR only when localized in the apoplast, and not when localized inside the plant cells (Tampakaki and Panopoulos 2000). Moreover, HrpZ was localized in the outer part of the plant cell after application of the partially purified protein on tobacco cell-suspension culture (Hoyos et al. 1996). HrpZ is able to insert in lipid bilayers and to form ion-conducting pores (Lee et al. 2001a and b), suggesting it would function on the host plasma membrane. HrpZ also has been shown to form multimers, up to octamers, in solution (Chen et al. 1998); therefore, it is possible that HrpZ could function as a membrane-associated complex. The harpin produced by *Erwinia* spp., encoded by the *hrpN* gene (Wei et al. 1992), was shown to affect plasma membrane ion channels in *Arabidopsis thaliana* suspension cells (El-Maarouf et al. 2001). Surprisingly, the association of harpins, both HrpZ and HrpN, with plant cell membrane seems to be a reversible event (Lee et al. 2001a; Pike et al. 1998). The harpins could be involved in the release of nutrients from the host cell or they could assist in the delivery of other bacterial proteins into the host cell. Several animal-pathogenic bacteria which utilize the TTSS produce proteins that form a protein translocation complex in the host cell plasma membrane (Blocker et al. 1999; Håkansson et al. 1996). A TTSS-harboring plant pathogen, *Xanthomonas campestris*, also secretes a membrane-inserting protein, HrpF, which binds to lipids and shows a pore-forming activity. HrpF is essential for the interaction with the host plant, and it has been suggested to have a translocation function (Büttner et al. 2002). Despite the lack of protein sequence homology, HrpZ might have an analogous function as a part of the TTSS.

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Whether the harpins interact with host proteins during the infection process is not known. Because *P. syringae* produces several harpin-like proteins, the function of HrpZ in pathogenesis is difficult to determine: a simple *hrpZ* mutant has no clear effect on HR induction in nonhost plants (Charkowski et al. 1998) or on virulence in host plants (Lee et al. 2001b). Yet HrpZ is produced by all but one of the *P. syringae* pathovars that have been characterized at the molecular level, and the sequence of this protein is highly conserved in the different pathovars, suggesting an unknown but possibly important pathogenesis-promoting function. HrpZ probably exerts its function as an integral membrane protein; therefore, the possible interactions between HrpZ and host proteins are likely to take place within the lipid environment. Thus, the putative interactions might be hidden by the lipids and hard to detect directly. We chose an indirect method to study the protein-binding characteristics of HrpZ involving the affinity of random peptides displayed on phage particles, and localized the peptide-binding site of HrpZ in a central region of the protein sequence using a defined set of the HrpZ mutant proteins.

RESULTS

HrpZ_{Pph} binds to a defined peptide sequence.

Phage clones carrying a peptide with affinity to HrpZ of *P. syringae* pv. *phaseolicola* race 6 (HrpZ_{Pph}) were enriched and isolated. The binding epitopes of 35 clones, confirmed by enzyme-linked immunosorbent assay (ELISA) to have a strong affinity for HrpZ_{Pph}, were sequenced. The sequences were found to encode only eight peptides (Table 1, 1 to 8), which are strikingly similar, all containing a hydrophobic amino acid motif with tryptophan and leucine, the consensus being W(L)ARWLL(G/L). The similarity of the peptide sequences suggests that HrpZ_{Pph} has a binding site for a protein and not just for any hydrophobic molecule. However, no sequences with a perfect match to the consensus peptide were found in the available sequence databases.

Peptide-binding site maps in the middle of the HrpZ_{Pph} sequence.

To locate the peptide-binding site within HrpZ_{Pph}, a library of randomly distributed 5-amino-acid (aa) insertion mutations on HrpZ_{Pph} was constructed. We developed an assay for determining the peptide-binding affinity of large amounts of semipurified harpin preparations, and screened the insertion mutants for loss of binding to the phage-displayed peptides 1 and 3 (Table 1). Of 276 mutants screened, we found 12 distinct non-binding mutants (Fig. 1A). Sequencing revealed that all of the inhibiting mutations resided in the central part of HrpZ_{Pph}, between amino acids 86 and 194. However, all mutations within this region, which might be called the peptide-binding domain, did not block binding: several noninhibiting insertion mutations were localized between the inhibiting mutations (Fig. 1B).

Interestingly, this domain includes a region of homology common to all of the HrpZ proteins and HrpN harpin of *Erwinia* and *Pantoea* spp. A short homology, within 22 aa, between HrpZ of *P. syringae* pv. *syringae* (HrpZ_{Pss}) and HrpN of *Erwinia amylovora*, was described previously by He and associates (1993), but we found that the homology extends over 69 to 85 aa (Fig. 2A). In addition to this conserved region, both HrpZ and HrpN have a domain with a low-complexity amino acid sequence rich in glycine, which is characteristic for harpins. In the HrpZ sequence, the glycine-rich domain is downstream of the conserved region; however, in HrpN, it is on the N-terminal side (Fig. 2B). Thus, HrpZ and HrpN seem to have two similar domains but in an inverse order.

HrpZ of *P. syringae* pv. *syringae* shows affinity to the HrpZ_{Pph}-binding peptides.

Harpins of other *P. syringae* pathovars, which have a different host range, and also HrpN harpin of a soft-rotting plant pathogen *E. carotovora* subsp. *carotovora*, were assayed to see if these proteins would have the same peptide-binding specificity as HrpZ_{Pph}. HrpZ_{Pss} bound the phage-displayed peptides number 1 and 3, although with lower affinity than HrpZ_{Pph}, whereas HrpZ of *P. syringae* pv. *tomato* strain DC3000 (HrpZ_{Pto}) did not bind (Fig. 3). For HrpZ_{Pph} and HrpZ_{Pto}, of which both semipurified and purified samples were available, the peptide-binding affinities of the preparations with different purity level were equal, indicating that impurities originating from *Escherichia coli* did not interfere with the peptide binding. Thus, the lower binding affinity showed by HrpZ_{Pss} probably was caused by the sequence differences between HrpZ_{Pss} and HrpZ_{Pph}, and not by other molecules present in the different harpin samples. HrpN had only a very weak interaction with the HrpZ_{Pph}-binding peptides, suggesting that this harpin, if binding to peptides, is likely to have a different binding specificity.

Antiserum raised against peptide 3 recognized plant proteins.

In order to find the protein that corresponds to the consensus peptide discovered via phage display, rabbits were immunized with the HrpZ_{Pph}-binding peptides 1 and 3, and the antisera were used to probe proteins of bacterial and plant origins. Chemical crosslinking experiments have indicated oligomerization of HrpZ_{Pph} (J. Lee and T. Nürnberger, *unpublished data*) and HrpZ_{Pph} itself has a tryptophan within the region where insertions inhibit peptide-binding; therefore, it is possible that oligomerization of the harpin might constitute the consensus peptide epitope. Thus, we first tested the peptide 3 antiserum for binding to immobilized HrpZ on Western blots, but the result was negative. However, when peptide number 3 was allowed to bind to the immobilized harpin in an overlay assay (far-Western) and then probed with the antiserum, the HrpZ band was revealed (data

Table 1. Phage-displayed peptides with a strong affinity to HrpZ of *Pseudomonas syringae* pv. *phaseolicola* race 6^a

Peptide no.	Amino acid sequence of the displayed peptide	Detection limit	Number of clones
1	PWLGLWLLGV	10 ⁴	1
2	PWLARWLLGV	10 ⁴	1
3	WARWLLTPA	10 ⁴	4
4	FVRWLLGV	10 ⁶	9
5	DTWARWML	10 ⁶	1
6	LGCWILPRL	10 ⁷	1
7	SWALWMLG	10 ⁷	14
8	WLGLMRW	10 ⁸	4
Consensus	W(L)ARWLL(G/L)	...	...

^a Detection limit denotes the number of phage particles required for a positive signal in enzyme-linked immunosorbent assay. Number of clones refers to the frequency in which the clones with an identical insert sequence were isolated. The central tryptophan of the HrpZ_{Pph}-binding peptides is shown in bold.

not shown). Thus, the result that the peptide 3 antiserum did not recognize the immobilized harpin was not caused by a damage of the harpin or lack of renaturation. As a whole, this indicates that the peptide-binding site is not mediating dimerization or oligomerization of HrpZ but, rather, an interaction of HrpZ with some other protein. Moreover, the peptide antisera also did not bind to protein samples of Hrp-induced *P. syringae* pv. *phaseolicola*, and an overlay (far-Western) with HrpZ did not reveal any interactions with the Hrp-induced bacterial proteins.

In addition to the proteins secreted by *P. syringae*, protein extracts of the host plant, bean, and four nonhost plants of *P. syringae* pv. *phaseolicola* were probed with the peptide anti-

sera. Peptide 3 antiserum recognized small (13- to 20-kDa) proteins in bean, tomato, *A. thaliana*, and parsley, but not in tobacco samples (Fig. 4A). Binding of the antibodies to the small proteins could be inhibited by pre-incubation of the peptide 3 antiserum (diluted 1:1000) with free peptide 3 (2 µg/ml). The complete blocking of antibody-binding by the peptide indicates that the small plant proteins are recognized by the same antibodies specific for peptide 3. To further characterize the peptide 3 cross-reacting proteins, the plant protein samples also were run on nondenaturing isoelectric focusing gels (IEF gels). The proteins found in bean, tomato, and parsley were very acidic, with pI 4.0 to 4.4, whereas the pI of the *A. thaliana* protein was 5.8 to 6.3. In protein sam-

Fig. 1. Binding of phage-displayed peptides to intact harpin and harpin mutants. **A**, Binding of phage peptide 3 to HrpZ of *Pseudomonas syringae* pv. *phaseolicola* race 6 (HrpZ_{Pph}) and the insertion mutants was determined by enzyme-linked immunosorbent assay with color development, absorbing light at 492 nm (A492). BL21, background, protein preparation from *Escherichia coli* with no harpin expression; ZPph, intact recombinant HrpZ from *P. syringae* pv. *phaseolicola*; D86-L263, HrpZ_{Pph} mutants carrying a 5-amino-acid (aa) insertion, named after the symbol and position of the amino acid affected by the insertion. **B**, Localization of the peptide-binding site of HrpZ_{Pph} by scanning insertion mutagenesis. Locations of the insertions that inhibit peptide-binding (Fig. 1A) are indicated on the sequence by black arrowheads and noninhibiting insertions are indicated by open arrowheads. HrpZ of *P. syringae* pv. *tomato* strain DC3000, which does not bind the peptides, contains a 24-aa-long extension not found in the other HrpZ proteins. The location of this extension is marked on the HrpZ_{Pph} sequence with a black ring. The region conserved in all the HrpZ and HrpN proteins is framed, and the hypersensitive response-inducing region is bold. The conserved repeats found in all HrpZ proteins are underlined.

ples from tobacco, no peptide 3 cross-reacting protein was detected on the IEF gel blots. The fact that the proteins seen in the native IEF blots were the same as the proteins detected from the sodium dodecyl sulfate–gel electrophoresis (SDS-PAGE) blots was confirmed by cutting the IEF gel into thin strips and extracting the proteins. These fractions were run on SDS-PAGE and then were blotted and probed with peptide 3 antiserum. This experiment was carried out with bean and *A. thaliana* protein samples and, in both cases, the result was positive: a 20-kDa protein of bean and a 13-kDa protein of *A. thaliana* were detected from the final blot.

HrpZ_{Pph} binds to an acidic, heat-sensitive protein of bean.

To verify whether the small acidic proteins identified above could directly interact with HrpZ_{Pph}, far-Western experiments were performed. The plant protein samples were run on nondenaturing isoelectric focusing gel, blotted onto polyvinylidene difluoride (PVDF) membrane, overlaid with HrpZ_{Pph}, and probed with HrpZ antiserum. Interestingly, the results showed binding of HrpZ_{Pph} to an acidic protein band of bean that was running in the gel at exactly the same position as the protein recognized by the peptide 3 antiserum. In-

stead, the acidic parsley proteins recognized by the peptide antiserum were not bound by HrpZ_{Pph} (Fig. 4B). With tomato and *A. thaliana* native protein blots, the result was similarly negative for harpin binding. Protein samples of the five plant species, originally containing both soluble proteins and membrane proteins on membrane micelles, were extracted further with chloroform-methanol mix to remove lipids and lipophilic compounds. The peptide 3 crossreacting proteins of bean, tomato, parsley, and *A. thaliana* partitioned into the aqueous phase, and their pI was not shifted by the extraction process (Fig. 4B), suggesting that major conformational changes had not occurred. The fact that both the peptide antiserum recognition and HrpZ_{Pph}-binding were not affected by the removal of lipids from the bean sample suggests that the interacting factors are hydrophilic proteins. Heating of the bean protein samples, with or without lipids, to 100°C for 10 min resulted in a pI change close to 5.0 of the peptide 3 crossreacting protein, and the loss of binding by HrpZ_{Pph} (data not shown). Taking this together with the fact that HrpZ_{Pph} did not bind to denatured proteins blotted from SDS-PAGE, we can conclude that the native fold of the bean protein is essential for the interaction with the harpin.

A

HrpZPpi	LDDLLTKQ-DGGAS- FS -EDDMPMLNKIA QFMD DNPAQ FPKP -----DSGSWV	
HrpZPss	LDDLLTKQ-DGGTS- FS -EDDMPMLNKIA QFMD DNPAQ FPKP -----DSGSWV	
HrpZPap	LDDLLTKQ-DGGSS- FS -EDDMPMLSKIA QFMD DNPAQ FPKP -----DSGSWV	
HrpZPph	LNDLLTKQ-DDGTR- FS -EDDMPMLKKIA EFMD DNPAQ FPKP -----DSGSWV	
HrpZPto	LDDLLTPSGEGGTT- FS -SDDMPTLEKVA QFMD DNKAQ FPTR -----DGGSWM	
HrpNPas	LNNIGTHS-DSSTRS FINK EDRALAREVG QFMD QY PETFGKP QYQKNADSAVKT DTKSWA	
HrpNPag	LNNIGTES-DSSTR FINK DDRSLAKEVG QFMD QY PEIFGKP QYQKTPYSDVKT DTKSWA	
HrpNEam	LNDIGTHS-DSSTRS FVNK GRAMAKE IGQFMD QY PEVFGKP QYQKGPQGEVKT DDKSWA	
HrpNEcc	LNNISTHN-DSPT RYFVD KEDRGMAKE IGQFMD QY PEVFGKPEY QKDNWQTAKQ DDKSWA	
HrpNEch	LSNVSTHV-DGNNRH FVD KEDRGMAKE IGQFMD QY PEIFGKPEY QKDGWSSPKT DDKSWA	
HrpZPpi	NELK--EDNFLDGDETA AFRSALDI I	125-193
HrpZPss	NELK--EDNFLDGDETA AFRSALDI I	124-192
HrpZPap	NELK--EDNFLDGDKTA AFRSALDI I	125-193
HrpZPph	NELK--EDNFLDGDETA AQFRSALDI I	125-193
HrpZPto	NELK--EDNGLDAQETA QFRSALDI I	148-217
HrpNPas	EALSQPDDGMPASME QFNKAKGI I	253-337
HrpNPag	EALSNPDDGMPASME QFNKAKGI I	253-337
HrpNEam	KALSKPDDGMPASME QFNKAKGI I	274-358
HrpNEcc	KALSKPDDGMPASME QFNKAKGI I	228-312
HrpNEch	KALSKPDDGMPASME QFNKAKGI I	211-295

B

Fig. 2. Domains of harpins HrpZ and HrpN. **A**, Sequence alignment of the homologous domains of HrpZ (*Pseudomonas*) and HrpN (*Erwinia* and *Pantoea*). This domain is located centrally in HrpZ protein sequence and near the N-terminus in HrpN. The alignment was produced by using CLUSTAL W (1.82) program with Blossum matrix. The most conserved amino acids are shown in bold, and the numbers following each sequence refer to the position of the amino acids shown, counted from the N-terminus of each protein. The abbreviations for species names are: Ppi, *Pseudomonas syringae* pv. *pisi*; Pss, *P. syringae* pv. *syringae*; Pap, *P. syringae* pv. *aptata*; Pph, *P. syringae* pv. *phaseolicola*; Pto, *P. syringae* pv. *tomato* (strain DC3000); Pas, *Pantoea stewartii* subsp. *stewartii*; Pag, *P. agglomerans* pv. *gypsophila*; Eam, *Erwinia amylovora*; Ecc, *E. carotovora* subsp. *carotovora*; and Ech, *E. chrysanthemi*. **B**, Functional domains of HrpZ of *Pseudomonas syringae* pv. *phaseolicola* race 6 (HrpZ_{Pph}) and HrpN of *E. amylovora* (HrpN_{Eam}). The type III secretion signal (SS) supposedly resides at the N-terminus, either in the amphipathic amino acid sequence or in the messenger RNA. Like most of the HrpN proteins, HrpN_{Eam} contains a polymethionine stretch (M). The region that is homologous between HrpZ and HrpN is crosshatched.

DISCUSSION

To find out whether HrpZ is interacting with proteins, in addition to the previously confirmed interaction with lipids, we studied the binding of random phage-displayed peptides to HrpZ_{Pph}. By using this method, we discovered that HrpZ_{Pph} binds peptides which contain tryptophan and leucine. The eight peptide sequences found could be aligned to define a consensus sequence W(L)ARWLL(G/L). The similarity of the HrpZ_{Pph}-binding peptides suggests that these peptides correspond to a protein epitope; a collection of unrelated (hydrophobic) sequences would, instead, indicate binding of HrpZ_{Pph} to any hydrophobic molecule, which could be either a protein or a lipid.

To localize the peptide-binding site of HrpZ_{Pph}, we performed scanning insertion mutagenesis of *hrpZ* gene. Screening the mutated proteins for peptide binding showed that inhibiting mutations all were located within the same region in the HrpZ_{Pph} sequence. This region is fairly large, covering a stretch of at least 108 aa; therefore, the peptide-binding site probably is formed by multiple amino acid residues distant from each other in the primary structure. This interpretation is supported by the presence of several noninhibiting mutations between the inhibitory mutations. The inhibiting insertion mutations may either block the peptide binding surface or interfere with the protein folding. It is noteworthy that these mutations reside in a region distinct from that deduced to act as an HR elicitor, which was previously located between amino acids 200 and 300 (Lee et al. 2001a). In accordance with this previous result, none of the peptide-nonbinding mutants were impaired in the ability to induce HR on tobacco leaves (data not shown). Hence, we identified protein–protein interaction here that probably is not involved in the induction of HR by HrpZ but might contribute to the compatible pathogen–host interaction. The maintenance of HrpZ and related sequences in various *P. syringae* genomes is highly indicative of an important function during the bacterial infection. A likely candidate is the pore-forming activity (Lee et al. 2001b). The full-length protein as well as either termini of HrpZ were capable of attaching to protein-free, lipid-coated silica beads (Lee et al. 2001b), but only the intact protein was able to form ion-conducting pores (*unpublished data*). Thus, attachment of HrpZ to lipids may be brought about by the termini, whereas the central domain may interact with a proteinaceous compound, as we have shown here. In conclusion, HrpZ_{Pph} seems to consist of several functional do-

main. The extreme N-terminal sequence probably contains the secretion signal for TTSS, the highly conserved central domain harbors a peptide binding site, and the N- and C-termini assist in membrane integration for pore formation. In addition, the accessible C-terminal region acts as an elicitor of defense responses in some resistant plants.

The variation in peptide-binding affinities shown by the HrpZ proteins of different *P. syringae* pathovars suggests that the harpins could be differentially interacting with a putative host-specific protein. Interestingly, HrpZ of the tomato pathovar did not show any binding affinity to the HrpZ_{Pph}-binding peptides. In addition to overall sequence divergence, HrpZ_{Pto} carries a 24-aa stretch not found in the other HrpZ proteins, and this extension is located within the region that is responsible for the peptide-binding in HrpZ_{Pph}. The extension sequence is homologous with the corresponding region of PopA1, a distantly related harpin produced by another tomato pathogen, *Ralstonia solanacearum* (Arlat et al. 1994). If the central region of HrpZ_{Pto} and PopA1 mediates an interaction with a host protein, one could assume that this protein would be different from the one interacting with HrpZ_{Pph}.

When plant protein samples were probed with antiserum raised against the HrpZ_{Pph}-binding peptide 3, the antiserum recognized small and acidic proteins in bean, tomato, parsley, and *Arabidopsis* leaf protein samples. The peptide 3-specific antibodies recognized these plant proteins on both SDS-PAGE blots and native IEF gel blots, whereas HrpZ_{Pph} did not bind to denatured proteins. Under nondenaturing conditions, HrpZ_{Pph} bound to a bean protein, as acidic as the peptide 3 crossreacting protein. This interaction was not disturbed but strengthened after extraction of the lipids from the bean sample, which further supports our conclusion that HrpZ_{Pph} binds to a host protein. Whether the protein recognized by the peptide antiserum is the same protein bound by the harpin is still unclear. Any proteins of tomato, parsley, and *Arabidopsis* having similar pI as the peptide 3 crossreacting proteins were not bound by HrpZ_{Pph}; therefore, we can conclude that a peptide 3-like epitope on a protein is not adequate for interaction with HrpZ_{Pph}. The epitope conformation could be incorrect or the surrounding protein structures could hinder the interaction. Therefore, the protein-binding site of HrpZ_{Pph} probably is not completely defined by the binding studies performed with short phage-displayed peptides. The fact that HrpZ_{Pph} tolerates some sequence variation in the bound peptides also suggests that the binding surface in the corresponding protein might be

Fig. 3. Binding of phage-displayed peptides 1 and 3 to different harpins. BL21, background; ZPph, ZPss, and ZPto, full-length HrpZ of *Pseudomonas syringae* pvs. phaseolicola, syringae, and tomato, respectively; HrpN, harpin of *Erwinia carotovora* subsp. *carotovora* SCC1. Data for both phages were combined of the results of four assays, each with at least four replicates of each protein sample. Each protein was applied on enzyme-linked immunosorbent assay plate at 50 ng/well.

formed by a structure larger than 8 aa. However, it seems that peptide 3 corresponds to an epitope present in a group of small and acidic plant proteins, and that HrpZ_{Pph} binds in a host-specific manner to a protein showing similar characteristics.

MATERIALS AND METHODS

Harpin expression and purification.

HrpZ_{Pph} and HrpZ_{Pto} were expressed by IPTG (isopropyl-β-D-thiogalactoside) induction in *Escherichia coli* strain BL21 (DE3) pLysS from vector pJC40 as previously described (Lee et al. 2001a and b). *hrpN* of *Erwinia carotovora* subsp. *carotovora* strain SCC1 (Rantakari et al. 2001) was amplified by polymerase chain reaction (PCR) (forward primer, 5'-GAGGAAA TCATATGCTTAATTCTCT-3' and reverse primer, 5'-CTCAA GCTTGCTTTAGCTGGA-3') and inserted into pJC40. HrpN was expressed from this construct, pJCHRPN, similarly as HrpZ_{Pph} and HrpZ_{Pto}, whereas HrpZ_{Pss} was expressed from pSYH10 (He et al. 1993). After 4 h of induction, the *Escherichia coli* cells were harvested and then frozen at -20°C, thawed, and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and lysozyme at 0.8 mg/ml). After incubation at room temperature for 15 min, the suspensions were heated at 100°C for 10 min and centrifuged at 16,000 × g at 4°C for 20 min. Although most of the other proteins were precipitated, HrpZ remained in the supernatant, which was used as a semi-purified harpin preparation. Cells expressing His-tagged HrpN were resuspended in 6 M guanidine HCl/0.1 M NaH₂PO₄/10 mM Tris-HCl, pH 8.0, and not heated but applied directly on Ni-NTA agarose (Qiagen, Hilden, Germany), then purified under denaturing conditions according to the manufacturer's protocol. For comparing the peptide-binding affinity, the His-tagged HrpZ_{Pph} and HrpZ_{Pto} also were purified by using Ni-NTA agarose, under native conditions.

Phage display.

Peptides binding to HrpZ_{Pph} were screened by using a phage display technique (Koivunen et al. 2001). Random peptides, a library of 10⁸ to 10¹⁰ different sequences, were inserted to the minor capsid protein PIII of a filamentous bacteriophage fUSE5. Three pools of such libraries with peptides displayed on the surface of the phage particles were screened. Pool I was composed of four libraries of linear 7- to 11-aa peptides and pool II of three libraries of 9- to 12-aa peptides crosslinked via terminal cysteines, and pool III contained a mix of five libraries with both linear and crosslinked peptides, 8 to 10 aa long. Microtiter plates were coated with HrpZ_{Pph}, 50 ng/well, and blocked with bovine serum albumin. The coated plates were incubated with the phage library pools, and the bound phage particles were used for infecting *E. coli* K91/kan cells as described by Koivunen and associates (2001). Phage particles were harvested from the *E. coli* culture supernatant and used for the next round of panning. After four rounds, HrpZ-binding phage clones were picked and the DNA inserts encoding the displayed peptides were amplified by PCR and sequenced. ELISA was used to confirm that the selected phages truly bind to HrpZ_{Pph}. The bound phage particles were detected with M13-specific monoclonal antibody and anti-mouse antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). Color reaction with *o*-phenylenediamine (OPD tablets; Sigma, St. Louis) allowed us to rate the HrpZ_{Pph} affinity of the phage-displayed peptides by reading absorbance at 492 nm.

HrpZ mutants.

The *hrpZ* gene of *P. syringae* pv. *phaseolicola* was mutagenized with a mini-Mu transposon, according to the protocol of the Mutation Generation System kit (Finnzymes,

Espoo, Finland). Insertion and subsequent deletion of the transposon created 15-bp insertions in the target gene, resulting in 5-aa insertions in the encoded protein. Mutated HrpZ proteins were expressed in *E. coli* BL21 similarly as was the intact protein. Each clone was grown in 1-ml volume and the cells were resuspended in 100 μl of lysis buffer. The HrpZ concentration of each semipurified protein preparation was determined by SDS-PAGE analysis. Samples of each preparation were applied on microtiter plates and screened for binding the phage-displayed peptides 1 and 3 by using ELISA as described above. To test whether the mutations affected HR-elicitation by HrpZ, 20-μg samples of the semipurified mutant proteins were injected into tobacco (cv. Samsun) leaves.

Peptide antisera.

Peptides number 1, 2, and 3 were synthesized on an ABIMED Economy Peptide Synthesizer (model EPS221; Langenfeld, Germany) according to the manufacturer's instructions. The quality of peptides was checked by MALDI-TOF mass spectrometry analysis. Rabbits were immunized with the synthetic peptides 1 and 3, which had been conjugated via the terminal cysteine residues to keyhole limpet hemocyanin (KLH kit; Pierce, Rockford, IL, U.S.A.). Peptide 1 antiserum was found specific for peptides 1 and 2, and peptide 3 antiserum was specific for peptide 3 in ELISA.

Extraction and immunoblotting of plant proteins.

To extract plant proteins, leaves of bean (*Phaseolus vulgaris* cv. Red Mexican), tomato (*Lycopersicon esculentum* cv. Agriset), tobacco (*N. tabacum* cv. Samsun), parsley (*Petro-*

Fig. 4. Western blots of proteins extracted from plant leaves. **A**, Plant proteins were run on a denaturing gel (sodium dodecyl sulfate–gel electrophoresis [SDS-PAGE]) and the blot was probed with peptide 3 antiserum. St, prestained broad range SDS-PAGE standards (Bio-Rad); Bn, bean; Tm, tomato; At, *Arabidopsis thaliana*; Ps, parsley; and Tb, tobacco leaf extract. Estimated sizes of the peptide 3 cross-reacting proteins are 20, 17, and 13 kDa for bean, tomato, and *Arabidopsis* proteins, respectively, and 19 and 17 kDa for the two parsley proteins. The 32-kDa bands in tomato and tobacco sample lanes are nonspecific, and the 65-kDa band in the bean sample is a KLH cross-reacting protein. **B**, Plant protein samples (20 μl) were run on a nonreducing isoelectric focusing (IEF) gel, blotted under non-reducing conditions, and probed with peptide 3 antiserum or HrpZ of *Pseudomonas syringae* pv. *phaseolicola* race 6. St, IEF standards pI 4.45 to 9.6 (Bio-Rad), of which the phycocyanin bands (pI 4.45 to 4.75) are shown; Bn and Ps, primary protein extracts from bean and parsley leaves; Be and Pe, bean and parsley primary protein extracts further extracted with chloroform/methanol to remove lipids.

selinum crispum cv. Hamburger Schnitt), and *A. thaliana* (var. Colombia) were homogenized in 50 mM MOPS-KOH buffer, pH 7.5, containing 5 mM EDTA, 330 mM sucrose, 0.1% polyvinyl pyrrolidone, 5 mM ascorbic acid, and 3 mM dithiothreitol (1 g of fresh leaves per 2 ml of buffer). The homogenate was filtered through Miracloth, and phenylmethylsulfonyl fluoride was added to 1 mM. The suspension was cleared by centrifugation at 13,000 rpm in a microfuge for 15 min, and the supernatant, containing water-soluble materials and microsomes with membrane proteins, was stored at -70°C . For preparing immunoblots, 10- μl samples of the plant protein extracts were run on SDS-polyacrylamide gel and blotted onto PVDF membrane. After blocking with 2% Tween 20 in Tris-buffered saline (TBS) the membranes were incubated overnight with peptide antiserum, diluted 1:1000 in 0.05% Tween/TBS. Second, the membranes were incubated with mouse monoclonal anti-rabbit antibody, conjugated to alkaline phosphatase (Sigma), and the bound conjugate was detected by using BCIP/NBT color-reaction (Promega Corp., Madison, WI, U.S.A.). Samples (10 to 20 μl) of the plant protein extracts also were run on nondenaturing IEF gels (IEF ready gels; Bio-Rad, Sundbyberg, Sweden), with pH ranging from 5 to 8 and 3 to 10, according to the manufacturer's protocol, and blotted in 0.7% acetic acid onto the PVDF membrane, which then was blocked with 3% casamino acids in 0.05% Tween/TBS. When performing far-Western analysis, the blot was incubated overnight with HrpZ_{Pph} at 0.5 $\mu\text{g}/\text{ml}$, and subsequently with rabbit HrpZ-antiserum, diluted 1:2000 in 0.05% Tween/TBS, for 4 h. To remove lipids, the plant protein extracts were extracted further with cold chloroform/methanol mix (2:1), one-fifth of the volume. The samples were mixed with vortex for 2 min, and centrifuged in a microcentrifuge at 10,000 rpm for 10 min at 4°C . The hydrophilic compounds, including water-soluble proteins, partitioned into the aqueous upper layer, whereas most proteins precipitated at the interface of the two phases, and lipophilic compounds partitioned into the organic solvent phase.

ACKNOWLEDGMENTS

The research performed at the University of Helsinki was financed by the Academy of Finland and the Helsinki Graduate School in Biotechnology and Molecular Biology. We thank E. Koivunen for providing the phage display libraries, I. Kilpeläinen for studying HrpZ by NMR spectroscopy, A. Rantakari for providing the *hrp* cluster of *E. carotovora*, J. Elster for technical assistance in the peptide synthesis, and E. von Roepenack-Lahaye for the MALDI-TOF quality control.

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