Elicitation of Hypersensitive Cell Death by Extracellularly Targeted HrpZ_{P_{sph}} Produced In Planta

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Accepted 9 August 2000.

The ability of the *Pseudomonas syringae* pv. *phaseolicola* harpin (HrpZ_{P_{sph}}) to elicit hypersensitive response was investigated in three *Nicotiana* genotypes. The *hrpZ_{P_{sph}}* gene was placed under chemical regulation (tetracycline induction) in TetR⁺ *Nicotiana tabacum* cv. Wisconsin 38 (W38) or was transiently expressed in *N. benthamiana* following infection with a PVX-derived vector and in three *Nicotiana* genotypes by agroinfiltration. The constructs were designed to express either the canonical form of harpin (HrpZ_{P_{sph}}) or an N-terminally extended version of the protein carrying the signal peptide portion of the tobacco pathogenesis-related protein PR1a (SP-HrpZ_{P_{sph}}). Stable transformants of *N. tabacum* cv. W38 did not develop necrosis upon induction with tetracycline, probably as a result of insufficient harpin accumulation. In contrast, *N. benthamiana* plants infected with the PVX constructs produced high concentrations of harpin in biologically active form, but only those expressing the secretable form of harpin developed necrotic symptoms. These symptoms were less severe than those caused by PVX::*avrPto*; however, they were accompanied by induction of *hsr203J*, a hypersensitive response-specific gene transcript. These results suggest that the plant cellular receptor(s) for harpin is extracellular.

Additional keywords: bacterial hypersensitive response elicitor, bean halo blight pathogen, *hrp-hrc* genes, type III secreted protein.

The interaction of plant-pathogenic bacteria with resistant host cultivars or nonhost plants often leads to a rapid, localized defense response termed hypersensitive response (HR), during which cells immediately surrounding the site of infection rapidly die (Klement et al. 1964). There is accumulating evidence that HR is a programmed cell death process (Greenberg et al. 1994; Wang et al. 1996) that is triggered following specific recognition between plant and pathogen proteins that are “functionally correspondent” and may interact physically at some early stage of the pathogen-plant en-

counter (Flor 1971). Following the initial discovery by Lindgren et al. (1986), it has been established that the elicitation of HR by a broad range of plant-pathogenic bacteria, including *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* spp., requires the functions of a suite of genes originally named *hrp* (HR and pathogenicity) (Bonas 1994) and more recently known as *hrp-hrc* (HR-conserved) (Bogdanove et al. 1996). These genes encode components of a novel protein secretion pathway designated type III secretion system (TTSS) (Van Gijsegem et al. 1993). This pathway is similar to those used by mammalian pathogens to secrete proteinaceous virulence factors such as Yops, Sips, and Ipas, some of which are delivered by the bacterial-secretion apparatus inside the mammalian host cell (Hueck 1998).

In plant pathogens the type III pathway is used to deliver proteins from the bacterial cytoplasm either to the culture medium or into the host cell cytosol. Some of these bacterial proteins can stimulate or interfere with host cellular processes (effectors), whereas others are involved in the secretion process, its regulation, or the translocation of the effectors through the host cell membrane. Effectors delivered by TTSS in plant-pathogenic bacteria elicit HR or contribute to pathogen virulence (Leach and White 1996). Effectors such as Avr proteins and harpins are intrinsically able to trigger HR in the absence of other bacterial proteins. Only the harpins and PopA1, however, elicit HR when supplied to the plant in purified form at relatively high concentrations (Arlat et al. 1994; He et al. 1993; Wei et al. 1992). In contrast, Avr proteins elicit HR only when they are expressed endogenously in the plant cells (Gopalan et al. 1996; Leister et al. 1996; McNellis et al. 1998; Scofield et al. 1996; Van den Ackerveken et al. 1996). These effectors all require the bacterial type III pathway to exit from the bacterial cell (e.g., harpins and PopA1) or to reach their appropriate destination in the host cell (e.g., Avr proteins). These findings, coupled with the fact that Avr proteins are generally not secreted by the bacteria in culture, led to the hypothesis that these proteins are delivered directly inside the plant cells via the type III secretion pathway and initiate cell death by interacting with cognate receptors. This hypothesis is in line with the intracellular nature of most “matching” R gene products (putative receptors) (De Wit 1997; Scofield et al. 1996; Tang et al. 1996). The intracellular delivery model is also in line with the ability of the type III system of mammal-

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ian pathogens to deliver virulence proteins inside the host cell (Hueck 1998).

The role of harpins in compatible and incompatible interactions is still enigmatic. Inferences about their site of action have been made from two types of studies. The HR induced by harpin from *Erwinia amylovora* (HrpN_{Ea}) and *Pseudomonas syringae* pv. *syringae* (HrpZ_{Pss}) is prevented by inhibitors of calcium influx and ATPase activity in tobacco (He et al. 1993; Popham et al. 1995). The HrpN_{Ea}-induced membrane depolarization, extracellular alkalinization, and potassium efflux in tobacco suspension culture cells has indicated that harpin has a pronounced effect on the plasmalemma, affecting H⁺-ATPase, ion channels, or membrane carriers (Pike et al. 1998; Popham et al. 1995). Moreover, Hoyos et al. (1996) reported that HrpZ_{Pss} binds to the outer portion of the cell surface of tobacco suspension culture cells and causes K⁺ efflux and extracellular alkalinization in tobacco cell suspension cultures but not in protoplasts. This work showed that HrpZ_{Pss} is not detected in the cytoplasm of fixed, permeabilized, suspension-cultured cells but in the periphery of plant cells with intact walls, suggesting that HrpZ_{Pss} possibly interacts with a component of the plant cell wall rather than the plasma membrane. These results do not necessarily rule out either possible interaction of HrpZ with the plasmalemma or internalization upon application to the extracellular medium of suspension cells due to the inherent limitations of the protoplast system and, possibly, to the low sensitivity of the immunofluorescence method.

To circumvent the limitations of these approaches and obtain further evidence concerning the mode and cellular site of harpin action, a fundamentally different approach was employed in the present study. Specifically, we investigated whether the HrpZ protein of *P. syringae* pv. *phaseolicola* (HrpZ_{Psph}) can elicit necrosis when expressed endogenously in plants. We expressed the protein in its canonical form and as a fusion with SP-PR1a, a plant protein signal peptide sequence. Our results showed that HrpZ_{Psph} elicits HR only when it is produced in sufficient quantity by the plant and, importantly, in a secretable form.

RESULTS

Expression of hrpZ_{Psph} in plants via *Agrobacterium*-mediated transformation.

Expecting that endogenously produced harpin may be lethal to the plant, we used the tetracycline inducible (Tet) expression vector system to achieve conditional expression of the hrpZ_{Psph} gene. The hrpZ_{Psph} coding frame was cloned in sense

and antisense orientation in the pBin-Hyg-TX vector. To direct the HrpZ_{Psph} protein to the plant cell exterior, the signal peptide (SP) sequence of the extracellular pathogenesis-related tobacco protein PR1a (SP_{Pr1a}) was fused in frame to the hrpZ coding sequence. Transgenic tobacco plants (TetR⁺, *Nicotiana tabacum* cv. Wisconsin 38 [W38]) were transformed by *Agrobacterium*-mediated leaf disk transformation. Thirty hygromycin-resistant, independent transformants were obtained for each transforming plasmid. Analysis of the transformants by Northern hybridization and immunoblotting revealed an hrpZ_{Psph}-specific transcript and the HrpZ_{Psph} protein after tetracycline treatment of leaves. The level of HrpZ_{Psph} protein did not vary significantly among different transformants. Contrary to our expectations, the primary transformants (T₀) and selfed progeny (T₁) did not reveal necrotic or other types of symptoms of any form during several days of observation after administration of tetracycline either via the root system or by injection in leaf mesophyll panels. Similar results were obtained by agroinfiltration of three *Nicotiana* genotypes (*N. tabacum* cultivars Xanthi and W38 and *N. benthamiana*). In these plants both the canonical and the secretable form of HrpZ_{Psph} were produced (not shown).

The failure of endogenously produced harpin to elicit necrotic symptoms could be a result of the protein not accumulating in sufficient quantity in the transgenic plants. Therefore, the amount of harpin produced in transgenic plants after tetracycline induction was quantified by immunoblotting and a rabbit polyclonal antibody prepared against HrpZ_{Psph}, which was purified from *Escherichia coli* and densitometry scanning of the bands (Fig. 1) with the National Institute of Health Image Program for Macintosh. Harpin accumulation was determined in total protein extracts from leaves after tetracycline induction from a reference plot that was based on known concentrations of purified protein. Two major immunoreactive

Fig. 1. Western blot analysis of HrpZ_{Psph}. Lane 1, Intercellular spaces of tobacco leaves after injection of *Pseudomonas syringae* pv. *phaseolicola*. The quantity corresponds to 0.2 cm² of leaf area. Lane 2, Leaves of *Nicotiana benthamiana* infected with PVX202::hrpZ_{Psph} in 0.076 cm² of leaf area. Lane 3, Leaves of transformed plants TetR⁺/HrpZ⁺ 4 days after tetracycline induction in hydroponic culture. The quantity corresponds to 0.2 cm² of leaf area. P = purified HrpZ_{Psph} from *Escherichia coli* (20 ng).

Fig. 2. Quantitative analysis of HrpZ_{Psph}. a, purified HrpZ_{Psph} needed for hypersensitive response (HR) elicitation. b, HrpZ_{Psph} produced in situ upon injection of *Pseudomonas syringae* pv. *phaseolicola* suspension into the intercellular spaces of tobacco leaf apoplast. c and d, HrpZ_{Psph} produced in tobacco transgenic plants TetR⁺/HrpZ⁺ and TetR⁺/SP-HrpZ⁺ expressing the canonical and the secretable form of HrpZ_{Psph}, respectively, after 4 days of induction with 1 mg of tetracycline per l in. hydroponic culture. e, HrpZ_{Psph} accumulated in leaves of *Nicotiana benthamiana* 12 days after infection with PVX202::hrpZ_{Psph}. Results represent mean values of two independent experiments. In each experiment, three leaves from different plants with the same treatment were used. Vertical axis is logarithmic and shows the quantity of HrpZ_{Psph} per square centimeter of leaf area. Intercellular space of well-expanded tobacco leaves was soaked with 10 µl/cm² of leaf area.

bands were observed in protein extracts from plants expressing the *hrpZ* gene. One corresponds to the full-length protein (upper band), and the other differs in apparent molecular mass by approximately 2 kDa. The origin of the lower band will be addressed later.

The quantity of harpin produced was compared with that produced by 10^7 CFU of *P. syringae* pv. *phaseolicola* per ml upon injection into the tobacco leaf apoplast and 100 µg of purified harpin per ml, the lowest concentrations able to elicit HR (Fig. 2). Harpin accumulation in the transformed plants (mass of protein/cm² of leaf) was 10-fold higher than the amount produced by bacteria in situ in standard HR tests. The bacteria, however, are most likely to deliver and/or specifically direct the elicitor to its correct cellular destination, possibly contributing other factors for HR development such as Avr proteins. In contrast, the transformed plants accumulated 50-fold less harpin than the quantity needed for HR elicitation when the protein is administered in purified form. The inability of harpin to elicit macroscopically visible HR in these plants may be attributed to insufficient quantity, improper

delivery, or lack of biological activity of the protein when it is produced endogenously in the plant.

PVX-mediated expression of HrpZ_{PspH} in *N. benthamiana*.

In an attempt to increase the intracellular accumulation of harpin, the *hrpZ*_{PspH} gene initially was cloned into the potato virus X-derived vector pPVX202, which permits high-level expression of heterologous genes in solanaceous plants. DNA of the recombinant virus PVX202::*hrpZ*_{PspH} was used to infect *N. benthamiana* leaves following the standard procedure (Sablowski et al. 1995). Control plants infected with PVX202 showed the characteristic chlorotic mosaic symptoms and leaf curling in the systemically infected leaves 12 to 15 days after infection. Plants infected with PVX202::*hrpZ*_{PspH} produced symptoms similar to those of the control plants over the same time period and did not develop any form of necrosis. The HrpZ_{PspH} protein was detected by Western blot in extracts from systemically infected leaves with PVX202::*hrpZ*_{PspH} 8 to 9 days after inoculation. In the same period, the expected sub-genomic viral transcripts were detected by Northern blot analysis by a riboprobe that was specific for the positive strand of PVX (Fig. 3). The quantity of harpin in these plants, quantified by densitometry scanning as described above, was substantially higher (100-fold) than in stably transformed *N. tabacum* cv. W38 TetR⁺/HrpZ⁺-expressing plants (Fig. 1). This amount was several-hundred-fold greater than that produced by bacteria injected into the leaf mesophyll at the lowest concentration needed to elicit confluent HR and roughly equal to the quantity of pure harpin able to elicit the reaction (Fig. 2).

Fig. 3. Northern blot analysis of mRNA extracted from *Nicotiana benthamiana* plants after inoculation with PVX and PVX202::*hrpZ*_{PspH}. The membrane was probed sequentially with a riboprobe specific for the positive strand of PVX (A) and with a ³²P-labeled DNA probe of the entire *hrpZ*_{PspH} sequence (B). Lanes: 1, total RNA extracted from leaves of PVX-infected plants 15 days after inoculation; 2 to 5, total RNA extracted from leaves of PVX202::*hrpZ*_{PspH}-infected plants at 8, 12, 16, and 20 days after inoculation, respectively. The position of various mRNA species are indicated on the left-hand margin. Autoradiographs were produced by exposure of panel A for 15 min, except lane 1, which was exposed for 1 h to detect the 6.4-kb transcript.

Fig. 4. Tobacco leaf showing the necrotic symptoms induced on tobacco leaves after injection of protein extracts from 1, healthy leaves of *Nicotiana benthamiana* (2 mg/ml); 2, systemically PVX202-infected leaves of *N. benthamiana* (2 mg/ml); 3, systemically PVX202::*hrpZ*_{PspH}-infected leaves of *N. benthamiana* (2 mg/ml); 4, purified harpin (100 µg/ml); 5, cell suspension of *Pseudomonas syringae* pv. *phaseolicola* (2×10^8 CFU/ml) in 10 mM MgCl₂. Leaf was photographed 24 h after infiltration under white light.

In light of the above results, it was important to establish whether the endogenously produced harpin in PVX202::*hrpZ*_{P_{sph}}-infected plants retained its HR-inducing activity. Total protein extracts were prepared from *N. benthamiana* leaves infected with either PVX202::*hrpZ*_{P_{sph}} or the vector alone. After ammonium sulfate precipitation and extensive dialysis, the extracts were injected into *N. tabacum* cv. Xanthi leaf mesophyll panels. Leaf panels injected with these extracts developed typical HR within 24 h, whereas similar extracts from healthy and PVX202-infected control leaves did not cause necrosis (Fig. 4). Similar results were obtained when the protein extracts were injected into *N. tabacum* cv. W38 and *N. benthamiana* leaves (not shown). These results showed that harpin produced in plants is biologically active. Therefore, the inability of endogenously produced harpin in PVX202::*hrpZ*_{P_{sph}}-infected leaves to elicit necrosis is not a result of insufficient quantity or a lack of intrinsic HR elicitor activity.

A most likely explanation of our findings is that the protein does not gain access to the appropriate cellular target(s) (e.g., outside the plant cell). This possibility was tested by infecting *N. benthamiana* plants with the recombinant virus PVX202::*SP-hrpZ*_{P_{sph}} encoding the secretable form of HrpZ. The plants developed mosaic symptoms in upper, noninoculated leaves 8 to 10 days after inoculation and numerous small necrotic lesions 3 to 5 days later. The symptoms occurred predominantly in the basal portion of the leaves (Fig. 5). Neither necrosis nor mosaic developed in the distal to the petiole portion of the leaves, indicating that the ensuing plant response was limiting the spread of the recombinant virus. Immunoblot analysis of total protein extracts from PVX202::*SP-hrpZ*_{P_{sph}}-infected plants revealed the presence of immunoreactive bands corresponding in size to SP-HrpZ_{P_{sph}} and HrpZ_{P_{sph}} (not shown), indicating that the protein was indeed produced and the SP extension was properly processed.

It is well documented that in planta expression of bacterial and fungal avirulence genes (e.g., *avrPto*, *avrRpt2*, and *avr9*) leads to necrosis (Hammond-Kosak et al. 1995; Kamoun et al. 1999; Mudgett and Staskawicz 1999; Tobias et al. 1999). Therefore, we compared the severity of necrosis caused by harpin and AvrPto in *N. benthamiana* infected with PVX202::*SP-hrpZ*_{P_{sph}} and PVX202::*avrPto* (Fig. 5). In contrast to the small necrotic lesions observed with PVX202::*SP-hrpZ*_{P_{sph}}, PVX202::*avrPto* elicited extensive necrosis over the entire basal portion of the systemically infected leaves 5 to 7 days after inoculation. Furthermore, expression of AvrPto caused death of the entire plant, which was not observed with harpin.

Necrosis caused by the secretable form of harpin is HR specific.

It has been established that *hsr203J* transcripts accumulate specifically in tissues undergoing HR following pathogen infection or harpin injection into leaf intercellular spaces (Pontier et al. 1994). To determine whether the necrosis observed in plants infected with PVX202::*SP-hrpZ*_{P_{sph}} is HR-specific, the induction of gene transcripts was examined by Northern hybridization. Total RNA extracts from *N. benthamiana* leaves systemically infected with PVX202::*hrpZ*_{P_{sph}} and PVX202::*SP-hrpZ*_{P_{sph}} and prepared at various times after inoculation were probed with an *hsr203J* probe (Fig. 6). Similar extracts from plants inoculated 6 h earlier with *P. syringae* pv. *phaseolicola* were also included in the Northern analysis. It proved experimentally difficult, however, to investigate the *hsr203J* transcript accumulation in plants infected with PVX202::*SP-hrpZ*_{P_{sph}} or PVX202::*avrPto* because of the transient induction of this gene and uncertainty about the kinetics of harpin accumulation in the PVX202-infected plants. Nevertheless, *hsr203J* was detected in PVX202::*SP-hrpZ*_{P_{sph}}-infected plants but not in those infected with PVX202

Fig. 5. Necrotic phenotypes of *Nicotiana benthamiana* plants inoculated with PVX202::*SP-hrpZ*_{P_{sph}} or PVX202::*avrPto*. *N. benthamiana* plants inoculated with PVX202::*SP-hrpZ*_{P_{sph}} (left) photographed 12 days after inoculation or with PVX202::*avrPto* (right) and photographed 7 days after inoculation.

(Fig. 6). The *hsr203J* transcript accumulation was observed prior to the onset of necrotic symptoms.

Origin of a truncated form of HrpZ_{PspH} produced in planta.

The expression of the *hrpZ_{PspH}* gene in planta generates two forms that differ in apparent molecular mass by approximately 2 kDa. We considered two possible scenarios for the origin of the lower molecular mass species. One possibility is proteolysis, based on the observation that other type III-delivered proteins are cleaved in bacteria (e.g., PopA1, HrpA, DspA, and AvrPphB) (Arlat et al. 1994; Gaudriault et al. 1997; Puri et al. 1996; Roine et al. 1997) or in planta (e.g., AvrRpt2, AvrB, and AvrPphB) (Gopalan et al. 1996; Mudgett and Staskawicz 1999; *our unpublished data*). Another possibility is alternative translation initiation on the basis that an internal ATG codon is present at position 16. Initially we examined whether harpin purified from *E. coli* or synthesized in vitro (TnT system) is cleaved in the presence of leaf protein extract. No cleavage or degradation was observed during 90 min of incubation at 37°C in the presence or absence of leaf protein extract, indicating that neither autoproteolysis nor plant protease-mediated cleavage was involved in the generation of the lower molecular mass form of harpin inside plant cells (Fig. 7A). We next examined whether the lower band may be a product of alternative translation initiation. The ATG codon at position 16 was changed to isoleucine by polymerase chain reaction (PCR) amplification with appropriately designed primers. The resulting construct (pT7-7/*hrpZ-M16I*) was expressed in vitro with the TnT-coupled rabbit reticulocyte lysate system. As expected, full-length HrpZ_{PspH} was produced, whereas the lower band was not detected (Fig. 7B). This indicates that the generation of the lower band seen in the plant is probably a product of internal translation initiation in plant cells.

DISCUSSION

The TTSS of plant-pathogenic bacteria serve as conduits to deliver HR effector proteins to the proper destination in the

plant host. Harpins remain the only type III-secreted proteins that are able to elicit HR when administered externally to plant tissues in purified form. The harpins described thus far in different phytopathogenic bacteria share several characteristics (Arlat et al. 1994; Bauer et al. 1995; He et al. 1993; Wei et al. 1992) that are also found in the HrpZ_{PspH} of *P. syringae* pv. *phaseolicola* NPS3121. It is glycine-rich, cysteine-lacking and heat stable. It also lacks a classical signal peptide and is secreted in culture media via the TTSS (*our unpublished data*). Hypotheses have been proposed to correlate the significance of these properties with the biological activity of harpins in the context of plant-bacterium interactions. One of the common characteristics of harpins is the lack of cysteine residues. It has been hypothesized that an unfolded state of harpins may facilitate their movement into the plant cell wall matrix rather than translocate through the Hrp pathway. Several bacterial Avr proteins thought to travel the type III pathway are relatively large and cysteine-rich and appear to interact with intracellular receptors. Direct evidence that harpins interact with extracellular receptors, however, is rather limited.

Our results provide strong evidence that HrpZ_{PspH} is able to elicit necrosis when expressed in planta in sufficient quantity and in a form that can be secreted to the plant cell exterior. We employed two different approaches to express harpin in plant cells: stable transformation and transient expression through PVX infection and agroinfiltration. Stable transformants expressing HrpZ_{PspH} or SP-HrpZ_{PspH} did not show any necrotic spots after tetracycline treatment in hydroponic culture, yet HrpZ_{PspH} protein was detected in leaves of transformants at levels 10-fold higher than those produced by bacteria in situ in standard HR tests with limit dilution of the inoculum. Under these conditions, cardinal molecular events associated with HR such as induction of *hsr203J* transcripts were not detected. Furthermore, agrobacteria expressing either the secretable or canonical form of harpin did not elicit HR following agroinfiltration in tobacco plants.

Assuming that the expression level of HrpZ_{PspH} in stably transformed and agroinfiltrated leaves was not sufficient, we employed another approach based on potato virus X to in-

Fig. 6. Induction of *hsr203J* in *Nicotiana benthamiana* leaves infected with PVX202::SP-*hrpZ_{PspH}*. Lane 1, Total RNA from leaves infected with *Pseudomonas syringae* pv. *phaseolicola* 6 h after injection. Lane 2, Healthy leaves. Lane 3, Systemically infected leaves with PVX 12 days after inoculation. Lane 4, Systemically infected leaves with PVX-202::SP-*hrpZ_{PspH}* and analyzed by Northern blot analysis. Each lane contained 30 µg of total RNA. **A**, Blot was hybridized with an *hsr203J* cDNA probe. **B**, Equal loading was verified before blotting by visualizing rRNA in a gel stained with ethidium bromide.

Fig. 7. **A**, Incubation of in vitro-translated harpin_{PspH} with and without total protein extracts from leaves of *Nicotiana benthamiana*. Lane 1, Input HrpZ_{PspH}. Lanes 2 and 3, HrpZ_{PspH} incubated for 90 min at 37°C with and without plant extracts, respectively. **B**, In vitro expression of wild-type HrpZ_{PspH} and M16I mutant derivative. Aliquots (5 µl) of the reactions were taken at 90 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. Lane 1, input HrpZ_{PspH}. Lane 2, M16I mutant. FL = full length HrpZ_{PspH}, and * = product of internal initiation. Translations of wild-type and mutant HrpZ_{PspH} were performed in the TnT rabbit reticulocyte lysate system in the presence of [³⁵S]-methionine with 1 µg each of pT7-7/*hrpZ* and pT7-7/*hrpZ-M16I* plasmids as templates.

crease the amount of harpin produced in the plant. The canonical form of HrpZ_{P_{sph}} failed to elicit HR, even though the protein was biologically active and accumulated at levels that were judged to be sufficient on the basis of the amount of pure harpin able to induce HR in the standard leaf-injection assay. These results clearly show that HrpZ_{P_{sph}} does not act inside the plant cells to signal HR. We can exclude the possibility that the lower molecular weight form of harpin detected in planta may interfere with full elicitor activity of the canonical protein. Published evidence (Alfano et al. 1996) shows that elicitor activity resides in multiple regions of HrpZ_{P_{ss}}. Furthermore, our own data (*unpublished*) shows that removal of the first 150 residues from HrpZ_{P_{sph}} did not alter its elicitor activity in tobacco leaf-injection assays.

In contrast to the results of the canonical form of harpin, necrosis was observed when the secretable form of the protein was expressed via the PVX vector. The induction of *hrp203J* confirmed that the necrosis was HR-specific. Additionally, detection of the transcript was not reproducible in our experiments. This could be explained by a combination of factors, including the asynchronous nature of the HR response elicited by PVX infection, the inability to predict the onset of transgene expression, and the temporal nature of *hrp203J* expression (Pontier et al. 1994). It is worth noting that the induction of this gene occurs prior to the onset of tissue necrosis. Our results show that overexpression of HrpZ_{P_{sph}} causes necrosis only when the protein is produced in sufficient quantity and secreted by the plant cells. This finding suggests that the putative receptor of HrpZ_{P_{sph}} is localized extracellularly, either in the plant cell wall or on the plant cell membrane. The existence of an extracellular receptor also has been suggested by the study of Hoyos et al. (1996), which proposed that harpin_{P_{ss}} may interact with a plant cell wall component(s) but not with the plasma membrane. The possibility that harpin may bind to the plasma membrane could not be discounted as a result of the technical limitations of the approach. For example, destruction of or conformational changes in harpin-binding sites may occur during enzymatic protoplast isolation or competition of cell wall peptides may be released by the enzymatic treatment with the harpin-binding sites. Our experimental approach yields results that are closer to the *in vivo* state because the integrity and functional relations between plant cell wall and plasmalemma are not disturbed. Our findings are further corroborated by a recent study (Lee et al., *unpublished data*), which showed that HrpZ_{P_{sph}} is stably integrated into artificial membranes and has pore-forming activity.

The necrosis caused by harpin expression showed differences compared with that caused by the expression of *avrPto*. The necrotic symptoms caused by harpin were milder and slower to appear compared with those caused by *AvrPto*. Several possibilities can be envisioned, such as that the secreted form of HrpZ_{P_{sph}} is expected after secretion to yield a polypeptide with an N terminus slightly different from that of the canonical harpin (substitution of four N-terminal amino acids of harpin with six different residues). The absence of the four canonical residues is unlikely to affect HR-elicitor activity for reasons mentioned earlier. The possibility that the six new residues might render the protein intrinsically less active, however, cannot be totally discounted. Note that a significant portion of SP-HrpZ_{P_{sph}} remains intracellular and therefore unavailable for interaction with external binding sites.

Whether the intracellular harpin can interfere with HR elicitation is an interesting but open question. Finally, it has been reported that HrpZ_{P_{ss}} and HrpN_{Ea} induce systemic acquired resistance (SAR) in cucumber and arabidopsis, respectively (Dong et al. 1999; Strobel et al. 1996). It is possible that the mild nature of the necrotic symptoms seen in our work may be the result of an early induction of SAR by endogenously produced HrpZ_{P_{sph}} that interferes with HR development.

The main conclusions from this study are that HrpZ_{P_{sph}} expressed endogenously in *N. benthamiana* causes HR only when it is produced in a secretable form via the PVX vector and in a sufficient quantity. The protein evidently interacts with an externally exposed receptor(s), which may be located in the plasmalemma or the cell wall. The nature of this receptor(s) and the molecular mechanisms underlying harpin perception by the plant cell remain to be elucidated.

MATERIALS AND METHODS

Plant material and bacterial strains.

N. tabacum cv. W38 TetR⁺ (Gatz et al. 1991), *N. tabacum* cv. Xanthi, and *N. benthamiana* were grown under controlled greenhouse conditions. Infiltration of tobacco leaves with bacteria, purified harpin, or plant protein extracts was carried out as described (He et al. 1993). *E. coli* strains and *A. tumefaciens* C58C1 carrying the disarmed Ti plasmid pGV2260 (Deblaere et al. 1985) were routinely grown on Luria-Bertani (LB) agar or broth at 37°C (Sambrook et al. 1989). *P. syringae* pv. *phaseolicola* (NPS3121) was grown in King's B broth at 30°C (King et al. 1954). For *in vitro* expression of *P. syringae* pv. *phaseolicola* *hrp* genes, cultures were grown in *hrp*-inducing fructose minimal medium of Huynh et al. (1989) at 30°C. Antibiotics were used at the following concentrations per milliliter: 100 µg of ampicillin, 100 µg of kanamycin, 100 µg of carbenicillin, 50 µg of rifampicin, and 50 µg of hygromycin.

Plasmid constructions.

The plasmid constructs were made according to standard molecular biological procedures as described by Sambrook et al. (1989).

pUC-A7-TX/SP. The coding region of the tobacco pathogenesis-related protein PR1a signal peptide portion (Pfitzner et al. 1987) was isolated by PCR amplification from *N. tabacum* cv. Xanthi genomic DNA. The primers (5'-GCCGCGGGTACCAAGCTTTCCTATAGTCATGGG-3' and 5'-CTCTGAGGGATCCTTGTTGAGAGTTTTGGGCACG-3') were designed to contain restriction sites (*Kpn*I and *Bam*HI, underlined) to facilitate cloning in the corresponding sites of the pUC-A7-TX vector (Roder et al. 1994). A 50-µl PCR of reaction mixture containing 10 pmol of each primer; 1 µg of *N. tabacum* genomic DNA; 10 mM KCl; 20 mM Tris-HCl, pH 8.8; 10 mM (NH₄)₂SO₄; 2 mM MgSO₄; 0.1% Triton X-100; 200 µM dNTPs; and 2 units of Deep Vent DNA polymerase (New England Biolabs Inc., Beverly, MA, U.S.A.) were subjected to 30 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C). The PCR product was digested with *Kpn*I and *Bam*HI and cloned into *Kpn*I/*Bam*HI-digested pUC-A7-TX.

pUC-A7-TX/*hrpZ*. The coding region of the *hrpZ*_{P_{sph}} gene from *P. syringae* pv. *phaseolicola* NPS3121 (343 codons) was

obtained from the pT7-7/*hrpZ* construct (Tampakaki et al. 1997) after digestion with *Nde*I and *Eco*RV. The protruding ends were filled in with Klenow DNA polymerase, and the digested fragment was cloned into the *Sma*I-digested pUC-A7-TX vector. The sequence of the *hrpZ*_{P_{sph}} gene will be published separately.

pUC-A7-TX/SP-*hrpZ*. The construct pUC-A7-TX/SP was digested with *Bam*HI, treated with mung bean nuclease to produce blunt ends, and digested with *Pst*I. The coding region of the *hrpZ*_{P_{sph}} gene was obtained from pUC-A7-TX/*hrpZ* after digestion with *Dde*I (Klenow fill-in) and *Pst*I. This fragment, which encoded the HrpZ_{P_{sph}} downstream from the fifth amino acid residue, was ligated to the above vector fragment. The resulting plasmid construct, pUC-A7-TX/SP-*hrpZ*, encodes a translational fusion between the PR1a signal sequence and the *hrpZ*_{P_{sph}} gene. The fused protein consists of 375 residues. The peptide expected after removal of the signal peptide (345 residues) includes six amino acids (QNSQQV), which substitute for the first four residues of harpin (MQSL).

pBIN-Hyg-TX/*hrpZ*. The *hrpZ*_{P_{sph}} gene, together with the modified CaMV35S promoter and the *ocs* transcriptional terminator, were excised from pUC-A7-TX/*hrpZ* as an *Eco*R-*Hind*III fragment and inserted into the corresponding sites of the binary vector pBin-Hyg-TX (Gatz, *personal communication*), conferring hygromycin resistance to transgenic plants.

pBIN-Hyg-TX/SP-*hrpZ*. As above, the chimeric gene SP-*hrpZ* was excised from pUC-A7-TX/SP-*hrpZ* as an *Eco*RI-*Hind*III fragment and inserted into the binary vector pBIN-Hyg-TX, which was linearized with *Eco*RI/*Hind*III.

PVX202::*hrpZ*. The fragment *Hpa*I/*Hind*III (Klenow fill-in) from construct pUC-A7-TX/*hrpZ* was cloned into the *Nru*I site of the viral expression vector PVX202 (Sablowski et al. 1995).

PVX202::*SP-hrpZ*. The fragment *Hind*III (Klenow fill-in) from the construct pUC-A7-TX/SP-*hrpZ* was cloned into the *Nru*I site of the viral expression vector PVX202.

PVX202::*avrPto*. The coding region of the *avrPto* gene was isolated by PCR amplification from the pDSK519/*avrPto* plasmid. The primers 5'-TGCTACTCGCGAGGGTATACGAA-TGGG-3' and 5'-GCCTCGCGAGTCGACATTATGACGCC-3' were designed to contain the restriction site *Nru*I (underlined) to facilitate cloning in the *Sma*I of pUC18 plasmid. The *avrPto* was excised from pUC18/*avrPto* as an *Nru*I fragment and inserted into the *Nru*I site of PVX202 vector.

pUC18/*hsr203J*. A portion of the coding region (nucleotides 1443 to 2041) of *hsr203J* corresponding to the published sequence (accession X77136) was isolated by PCR amplification from the genomic DNA of *N. tabacum* cv. Xanthi using primers 5'-CGCGGATCCGGCTGGCTTAGAG-TTTTC-3' and 5'-TCCGGGATCCCTCCGATAGGACCGCA-CG-3'. Both primers were designed to contain the *Bam*HI restriction site (underlined) to facilitate cloning into the corresponding site of the pUC18 plasmid.

pT7-7/*hrpZ*-M16I. The plasmid pT7-7/*hrpZ* was used to mutagenize the methionine codon at position 16 (based on the *hrpZ*_{P_{sph}} sequence, *our unpublished data*) to an isoleucine codon in accordance with the protocol described by Fisher and Pei (1997). The mutagenesis was performed by PCR amplification with the primers 5'-CGATCGCGCTCGTTCTGAT-CCGTCC-3' (forward, mutagenic primer) and 5'-ACGGGCT-TTCGAGCGTGCTGCTG-3' (divergent, nonoverlapping pri-

mer). The mutagenic primer contains the mutant codon for isoleucine (italics) and a *Pvu*I restriction site (underlined). The mutation was confirmed by *Pvu*I digestion and sequencing.

Plant transformation.

Healthy, well-expanded leaves from 6-week-old plants of *N. tabacum* cv. W38 TetR⁺ were used for *Agrobacterium* transformation by leaf disk method as described by Horsch et al. (1988). The binary plasmid constructs were introduced into *Agrobacterium tumefaciens* by triparental conjugation (Rogers et al. 1988). Colonies were selected on LB medium with 1.5% Bacto agar, 50 µg of rifampicin per ml, 100 µg of carbenicillin per ml, and 100 µg of kanamycin per ml. The transconjugants were used to transform *N. tabacum* cv. W38 TetR⁺ plants. Hygromycin-resistant transformants were grown in vitro on Murashige-Skoog media (Murashige and Skoog 1962) with appropriate hormone regimes for shoot and root formation and subsequently maintained in hydroponic culture under controlled conditions (25°C, 16-h photoperiod) or in pots in the greenhouse.

Protein extraction and analysis.

Leaves were immediately frozen in liquid nitrogen after collection and broken with a mortar and pestle. Total soluble protein was extracted in a buffer containing 200 mM Tris-HCl, pH 8; 1 mM EDTA; 5 mM DTT; 0.5 mM PMSF; 10 µM leupeptin; 10% glycerol; and 0.25% Triton X-100. Samples were incubated with shaking on ice for 30 min and centrifuged (14,000 rpm, 15 min, 4°C). The supernatants were then collected and kept at -20°C in small aliquots.

Immunoblot analysis.

Accumulation of the *hrpZ*_{P_{sph}} gene product was assayed in hygromycin-resistant, regenerated plantlets by quantitative immunoblotting. Soluble leaf protein was extracted, separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to the nitrocellulose membrane by standard procedures. The membranes were blocked and incubated with an anti-HrpZ_{P_{sph}} antibody (1:20,000) and developed with an alkaline phosphatase-conjugated antibody with nitroblue tetrazolium and 5-bromo-4-chloro-3-iodyl phosphate, in accordance with the supplier's instructions. To detect PVX coat protein accumulation, the membranes were incubated with an anti-PVX antibody (1:2,000 dilution) and developed as above.

Plant nucleic acid extraction and analysis.

Plant DNA was isolated with the method described by Rogers and Bendich (1988). Total RNA was isolated from tobacco leaf tissues with Tri Reagent (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.). Leaves were frozen in liquid nitrogen immediately after harvesting and kept at -80°C for further extraction. Thirty micrograms of total RNA from each sample was loaded on a denaturing 1.2% formaldehyde-agarose gel, separated by electrophoresis, and transferred onto a nylon filter (Hybond-N, Amersham, Arlington Heights, IL, U.S.A.). Following UV cross-linking, the filters were prehybridized for 2 h at 65°C in 0.5 M phosphate buffer, pH 7.2, containing 7% SDS, 1% bovine serum albumen, and 1 mM EDTA (Church and Gilbert 1984). The filters were hybridized in the same buffer with cDNA and labeled with ³²P-dATP and ³²P-dCTP by random priming procedure (Sambrook et al. 1989). The

membranes were washed in 0.4 M phosphate buffer, pH 7.2, containing 5% SDS and 1 mM EDTA at 65°C.

Tetracycline induction.

Transgene induction in *N. tabacum* cv. W38 TetR⁺ primary transformants (T₀) or T₁ progeny was accomplished by supplying tetracycline at a concentration of 1 mg per liter, either by vacuum infiltration of detached leaves or in hydroponic culture in Hoagland buffer as described by Gatz et al. (1991).

PVX-inoculations.

Four-week-old *N. benthamiana* plants were inoculated with the viral expression vector PVX202, PVX202::hrpZ_{P_{spH}}, or PVX202::SP-hrpZ_{P_{spH}} by gently rubbing leaves dusted with Carborundum, as described by Sablowski et al. (1995). Two leaves of each plant were inoculated with 5 to 10 µg of Qiagen-purified (Chatsworth, CA, U.S.A.) DNA dissolved in 30 µl of 50 mM sodium phosphate buffer, pH 7. Two weeks later, young, systemically infected leaves were harvested and immediately homogenized in the same buffer or stored at -80°C for protein and RNA extraction and plant inoculations.

In vitro transcription and translation.

Wild-type HrpZ and the mutant M16I were expressed from the pT7-7 templates by the TnT T7 rabbit reticulocyte lysate-coupled transcription-translation system (Promega, Madison, WI, U.S.A.) in the presence of [³⁵S] methionine (final concentration, 1 mCi/ml) (Amersham), in accordance with the manufacturer's instructions. Reactions were incubated at 30°C for 90 min. Aliquots of the reactions (5 µl) were run on 12% SDS-polyacrylamide gels, which were dried and analyzed with autoradiography.

ACKNOWLEDGMENTS

We thank C. Gatz for the plasmids of the tetracycline-regulated system and TetR⁺ tobacco seeds, B. J. Staskawicz for the pDSK519/avrPto plasmid, D. C. Baulcombe for the PVX vector, E. Krabovitis for the PVX antiserum, and S. Mavrakis for greenhouse assistance. Financial support was provided in part by research contracts from the European Community (BIO4-97-2244) and the Secretariat for Research and Technology of Greece (EPET II-233 and PENED ED3L3).

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