Molecular Determinants Required for the Avirulence Function of AvrPphB in Bean and Other Plants

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The avirulence gene *avrPphB* from *Pseudomonas syringae* pv. *phaseolicola* determines incompatibility, manifested as a hypersensitive reaction (HR), on bean cultivars carrying the *R3* resistance gene and also confers avirulence on other plants. The AvrPphB protein carries an embedded consensus myristoylation motif and is cleaved in bacteria and certain plants to yield fragments of about 6 and 28 kDa. We investigated plant recognition and type III translocation determinants in AvrPphB by constructing three N-terminally truncated and two site-directed mutants carrying substitutions in the conserved G63 residue of the myristoylation motif, which lies adjacent to the proteolytic cleavage site. The peptides were either delivered to plant cells by pseudomonads or were expressed transiently in planta via the *Agrobacterium tumefaciens* or *Potato virus X*. The 63 amino terminal residues were required for type III-mediated translocation from *Pseudomonas* strains to the plant, but were partially dispensable for effector recognition following in planta expression. Substitution of the G63 residue resulted in differential HR phenotypes in two different *R3* cultivars of bean and abolished effector processing in *Pseudomonas* strains. *Agrobacterium*-mediated expression of the mutant proteins elicited HR in resistant bean hosts and in tomato but elicited no reaction in *Nicotiana* species.

Additional keywords: pathogenicity, receptor, signaling, type III secretion.

Race- and cultivar-specific resistance to halo-blight in bean (*Phaseolus vulgaris* L.) is based on gene-for-gene interactions involving five genes for resistance (*R*) in the host and five matching genes for avirulence (*avr*) in the pathogen, *Pseudomonas syringae* pv. *phaseolicola* (Jenner et al. 1991; Mansfield et al. 1994). Three *avr* genes from this pathogen, those matching *R1*, *R2*, and *R3*, have been cloned and sequenced (Jenner et al. 1991; Mansfield et al. 1994; Tsiamis et al. 2000). One of these, *avrPphB*, matches *R3* from *Phaseolus vulgaris*. Identical alleles of *avrPphB* occur in races 3 and 4 of *P. syringae* pv. *phaseolicola*, which cause a characteristically rapid hypersensitive reaction (HR) in bean cultivars with *R3* (Jenner et al. 1991; Mansfield et al. 1994). No DNA sequences hybridizing to probes for *avrPphB* are found in strains of *P. syringae* pv. *phaseolicola* that are virulent on *R3* cultivars, indicating that *avrPphB* is not generally required for pathogenicity in the

halo-blight bacterium. *avrPphB* also confers avirulence to pea, soybean, and *Arabidopsis* after transfer to appropriate bacterial pathogens that are virulent on these hosts (Fillingham et al. 1992; Pirhonen et al. 1996; Simonich and Innes 1995). In *Arabidopsis*, the matching *R* gene, *RPS5*, has been cloned (Simonich and Innes 1995; Warren et al. 1999). Determination of specificity in such a wide range of plants has now been reported for other bacterial *avr* genes from other pathovars of *P. syringae*, notably *avrB*, *avrRpm1*, and its homologue *avrPphA1.R2* (Dangl et al. 1992; Fillingham et al. 1992; Grant et al. 1995; Vivian et al. 1997).

Accumulating evidence suggests that bacterial avirulence (Avr) proteins are delivered inside the host cells through the *hrp/hrc*-encoded type III secretion system (TTSS) and are subsequently recognized either directly or indirectly by the corresponding *R* gene products. This hypothesis is based primarily on studies showing that several Avr proteins elicit resistance gene-dependent cell death when they are expressed in planta (Galan and Collmer 1999; Gopalan et al. 1996; Leister et al. 1996; Van den Ackerveken and Bonas 1997). However, the physical transfer of Avr proteins has not been demonstrated directly. In most cases, the final destination of Avr proteins following delivery to the plant cell is not clear, exceptions being the AvrBs3 family members of *Xanthomonas*, which evidently are targeted to the plant nucleus via eukaryotic nuclear localization signals (Van den Ackerveken et al. 1996; Yang and Gabriel 1995; Zhu et al. 1998, 1999).

A subset of *P. syringae* type III effectors, AvrB, AvrC, AvrRpm1, AvrPto, and AvrPphB, contain potential myristoylation motifs at their amino termini. Protein N-myristoylation is responsible for the localization of many eukaryotic proteins to the plasma membrane or to the other intracellular membranes (Resh 1994, 1999; Towler et al. 1988). Although N-myristoylation has only been observed in eukaryotes, myristoylation and other types of acylation of Avr effectors occur inside plant cells and mediate their proper localization to the plant plasma membrane, where molecular recognition events presumably take place (Nimchuk et al. 2000; Shan et al. 2000). AvrPphB is unusual among this group of effectors in two respects. First, its consensus N-myristoylation site is embedded in the sequence (G₆₃CASSGV₇₀) rather than being amino-terminal, and substitution of glycine at position 63 to an alanine abolishes membrane association and avirulence function in *Arabidopsis* Col-0 plants, which possess the matching *RPS5* gene (Nimchuk et al. 2000). Second, the protein is cleaved in *Escherichia coli* between residues K62 and G63, yielding a 28 kDa and a 6 kDa species (Puri et al. 1997). Similar processing of AvrPphB occurred in *P. syringae* pv. *phaseolicola*, as well as in *Arabi-*

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dopsis, when the protein was expressed endogenously. The exact site and the role of processing in Type III-mediated translocation or in planta recognition have not yet been elucidated. However, Nimchuk and associates (2000) proposed that cleavage potentially exposes the G63 residue within the putative myristoylation motif, enabling subsequent modification of the 28-kDa species and membrane association of the truncated protein. It is interesting that the myristoylation motif borders with the proteolytic cleavage site determined in *E. coli*. However, it has not been determined whether the processing of AvrPphB affects R receptor recognition or translocation via the bacterial type III apparatus.

The processing of AvrPphB is of further interest in light of current knowledge concerning the translocation of effector proteins via the type III secretion apparatus. Type III effectors lack a classical signal peptide and other common sequence features. However, recent studies showed that the TTSS machinery recognizes effector proteins by two signals in their amino termini: a secretion signal and a translocation signal (Sory et al. 1995). Secretion signals have been mapped in several Yop proteins to sequences encoding the amino-terminal 15-amino acid residues (Cornelis 2000; Cornelis and Van Gijsegem 2000) or their codons (Anderson and Schneewind 1997; Anderson et al. 1999). Translocation signals are located within the amino-terminal 50- to 100-amino acid residues of several Yop effectors

(Plano et al. 2001), which are required for binding to their cognate chaperone. Although most Avr proteins are not secreted extracellularly by bacteria grown under *hrp*-inducing conditions, a subset of Avr proteins can be secreted to some degree under certain conditions by strains expressing either homologous or heterologous type III-secretion systems (Ham et al. 1998; Van Dijk et al. 1999). Recently, it was suggested that the first 15 codons of *avrPto* and the first 18 codons of AvrBs2 might carry a potential m-RNA targeting signal (Anderson et al. 1999; Mudgett et al. 2000).

To define the sequence features of AvrPphB that are required for translocation via the TTSS to the host cells and for effector-host cell recognition, we carried out deletion and site-directed mutagenesis studies. Transient expression of both the full-length AvrPphB protein and the 28-kDa species lacking the 63 N-terminal residues inside plant cells in bean cultivars, as well as in several nonhost plant species, was achieved by agroinfiltration and *Potato virus X* (PVX) infection, respectively. Our results clearly show that deletion of the 63 N-terminal amino acids only partially reduced elicitor activity after in planta expression but completely abolished avirulence function in *P. syringae* pv. *phaseolicola*. Our results clearly show that the 63 N-terminal amino acids are required for full elicitor activity as well as for type III translocation. Site-directed mutagenesis further showed that processing of AvrPphB appears to be important for translocation in a host-dependent manner and that the putative myristoylation motif is differentially required for elicitation of the HR in different plant species.

RESULTS

The bacterial TTSS is required for the expression of AvrPphB-dependent avirulence.

To determine whether the type III-secretion apparatus of *P. syringae* pv. *phaseolicola* is involved in AvrPphB delivery to the plant, we introduced a broad-host range plasmid expressing the AvrPphB protein under the *lac* promoter into a wild-type Race 6 strain (*P. syringae* pv. *phaseolicola* R6) and in a TTSS-deficient mutant that carries a nonpolar deletion in the structural gene for the Hrp pilus subunit (R6 Δ *hrpA*). *P. syringae* pv. *phaseolicola* R6 is virulent on all bean cultivars used in this study, as previously reported (Taylor et al. 1996). The R6 Δ *hrpA* mutant was neither pathogenic nor did it elicit HR in bean, regardless of the presence or absence of the *R3* gene (Lee et al. 2001). The *P. syringae* pv. *phaseolicola* R6 transconjugants expressing *avrPphB* (R6-*avrPphB*) caused brown necrosis, a manifestation of HR, in leaves as well as in pods of bean cultivars in an *R3*-dependent manner, while the pilus-deficient transconjugants (R6 Δ *hrpA*-*avrPphB*) caused neither water-soaking nor HR (Fig. 1A). Western blot analysis of total cell extracts with a polyclonal AvrPphB antibody showed that the protein was expressed and processed normally in the *P. syringae* pv. *phaseolicola* bacteria, indicating that constitutive expression of AvrPphB or the Δ *hrpA* mutation does not interfere with the processing of the protein in *P. syringae* pv. *phaseolicola* (data not shown). The results clearly show that a functional *hrp*-encoded TTSS is required for AvrPphB delivery from the bacteria to the plant.

The 63 N-terminal residues are partially dispensable for the avirulence function of AvrPphB.

We were interested in defining the role of AvrPphB processing in the bacterial host and in defining the structural requirements for delivery and host recognition of the effector. Initially, deletion mutants of *avrPphB* were constructed by introducing an ATG codon before codons 64 and 110, simultaneously removing the corresponding N-terminal regions (mutant

Fig. 1. A, Reaction phenotypes in pods of cultivars Tendergreen (TG) and A43 3 days after inoculation with (1) R6, (2) R6 containing the vector, (3) R6 expressing *avrPphB*, (4) R6 Δ *hrpA*, and (5) R6 Δ *hrpA* expressing *avrPphB*. **B,** Reaction phenotypes in pods of cvs. TG, A43, and A52 3 days after inoculation with (1) Race 6 containing the vector, (2) expressing *avrPphB*, (3) NA63, (4) NA109, (5) G63A, and (6) G63E. Cvs. TG and A43 are resistant to *Pseudomonas syringae* pv. *phaseolicola* (*avrPphB*) strains, cv. A52 is susceptible.

designations NΔ63 and NΔ109, respectively). The mutants were cloned in a broad-host range vector and were introduced in *P. syringae* pv. *phaseolicola* R6. Next, transconjugants expressing the wild-type AvrPphB protein (R6-*avrPphB*), the two deletion mutants (R6-NΔ63 and R6-NΔ109), or the empty vector (R6-EV) were used to inoculate pods of resistant and susceptible bean cultivars, and the symptoms were scored 3 days after inoculation. As expected, R6-EV transconjugants developed typical water-soaked lesions in pods of bean cultivars possessing or lacking the *R3* gene. The R6-*avrPphB* transconjugants caused brown necrotic lesions that are characteristics of the HR in pods of cvs. Tendergreen (TG) and A43, which carry the *R3* gene. In contrast, R6-NΔ63 and R6-NΔ109 transconjugants developed water-soaked lesions on pods of these cultivars (Table 1, Fig. 1B). To rule out the possibility that the loss of avirulence activity in these mutants is a result of reduced stability and abundance of the protein, total cell extracts, as well as the culture mediums of R6-*avrPphB*, R6-NΔ63, and R6-NΔ109 transconjugants grown on *hrp*-inducing medium, were examined by Western blot analysis using the polyclonal AvrPphB-specific antibody. Both mutant polypeptides accumulated normally in *P. syringae* pv. *phaseolicola* R6, as did the full-length protein, but neither these, nor the full-length AvrPphB or its 28-kD form could be detected in the culture medium (data not shown). These results suggest that the 63 N-terminal amino acids of AvrPphB are necessary either for its translocation through the TTSS or for recognition by the cognate plant receptor.

In planta recognition determinants of AvrPphB.

To investigate whether the 63 N-terminal residues of AvrPphB were required for effector recognition, deletion mutants NΔ63 and NΔ109 were transferred to a binary *Agrobacterium* vector and were tested for their ability to cause HR in different plant species by agroinfiltration. The NΔ63 mutant, but not NΔ109, triggered weaker and delayed necrosis in comparison with the full-length protein in bean cultivars carrying the *R3* gene (Table 1, Fig. 2A). Neither the mutants nor the wild-type protein elicited HR on bean cultivars lacking the *R3* gene, indicating that the necrosis caused by the wild type and the NΔ63 effector is *R3* gene specific. Similar weak HR activity was observed on three other nonhost plant species, namely, tomato (cvs. Rio Grande 76S and 76R), tobacco (cvs. Xanthi and W38), and *Nicotiana benthamiana* (Table 1, Fig. 2B). The presence of *R3* gene homologues in *N. benthamiana* was also investigated by introducing the broad-host range plasmid ex-

pressing the wild-type *avrPphB* and the compatible cosmid pPL6, which carries the entire *hrp* cluster from *P. syringae* pv. *phaseolicola* strain NPS3121 (Lindgren et al. 1986) into the nonpathogenic bacterium *P. fluorescens*. Transconjugants carrying both plasmids elicited HR, while those carrying the cosmid or the plasmid expressing *avrPphB* alone did not elicit any reaction (data not shown). Thus, *N. benthamiana* evidently carries a matching resistance gene for *avrPphB*.

Conceivably, the weaker HR phenotype of the NΔ63 mutant may be due to decreased stability or lower accumulation of the truncated proteins in the plant, or both, or to the removal of molecular determinants involved in recognition by the cognate R receptor. However, we were not able to detect AvrPphB or the mutant proteins in agroinfiltrated leaves of tomato, bean, and tobacco by Western blot analysis, which suggests that all forms of the effector are rapidly degraded in planta, as previously reported for AvrPphB and other Avr effectors in *Arabidopsis* (Nimchuk et al. 2000).

To compensate for possible reduced accumulation of the NΔ63 protein in the agroinfiltrated leaves, we sought to increase its accumulation level by expressing the truncated NΔ63 gene from a PVX vector, which in our previous work (Tampakaki and Panopoulos 2000) gave substantially greater quantities of another HR elicitor, HrpZ_{PspH}, in planta. As PVX cannot infect bean, we used *N. benthamiana* and tomato as resistant plant hosts. Plants inoculated with the empty PVX vector developed chlorotic mosaic, the typical symptoms of PVX infection, in the upper noninoculated leaves 1 week after inoculation. In contrast, plants infected with the recombinant virus PVX202::*avrPphB* developed necrotic lesions in the upper noninoculated leaves within 5 to 7 days (Fig. 2C), whereas the NΔ63 mutant caused delayed necrosis in both plant species (Table 1). However, as with agroinfiltrated leaves, we could detect neither the wild-type nor the mutant protein in PVX-inoculated leaves.

It is possible that protein instability in planta may have been responsible for the weaker HR elicitor activity of NΔ63, but another possible explanation may be the absence of the G63 residue (Fig. 3). Nimchuk and associates (2000) hypothesized that the full-length protein (35 kDa) is cleaved in planta to produce an HR-active 28-kDa species and that this cleavage exposes the G63 residue for possible myristoylation. To investigate the possibility that the G63 residue was essential for the HR-elicitor activity of the 28-kDa form of AvrPphB, we used another deletion mutant, NΔ62, in which an ATG codon had been introduced just before the G63 codon (Stevens et al.

Table 1. Hypersensitive reaction (HR) phenotypes in bean and other plants^a

Plant and cultivar ^b	<i>R3</i> gene allele	Delivery vehicle ^c	AvrPphB ^d	NΔ62	NΔ63	NΔ109	G63A	G63E
Bean cv. Tendergreen (TG)	+	<i>Agro</i>	+++ ^e	++	++	–	+++	+++
		<i>PspH</i>	+++	–	–	–	–	–
Bean cv. A43	+	<i>Agro</i>	+++	++	++	–	+++	–
		<i>PspH</i>	+++	–	–	–	++(+) ^f	++(+)
Bean cv. A52	–	<i>Agro</i>	–	–	–	–	–	–
		<i>PspH</i>	–	–	–	–	–	–
Bean cv. Canadian Wonder (CW)	–	<i>Agro</i>	–	–	–	–	–	–
		<i>PspH</i>	–	–	–	–	–	–
Tomato	Unknown	<i>Agro</i>	+++	++	++	–	+++	+++
<i>N. benthamiana</i>	Unknown	<i>Agro</i>	+++	++	++	–	–	–
Tobacco	Unknown	<i>Agro</i>	+++	++	++	–	–	–

^a Induced by AvrPphB deletion and site-directed mutant polypeptides delivered by *Pseudomonas syringae* pv. *phaseolicola* or *Agrobacterium*-mediated transient assay.

^b Known genes for resistance to *P. syringae* pv. *phaseolicola* in bean cultivars are as follows: TG, *R3*; A43, *R2*, *R3*, *R4*, *R5*; A52, *R4*; CW, none.

^c *Agro* = *Agrobacterium*-mediated transient expression of corresponding gene; *PspH* = *P. syringae* pv. *phaseolicola* Race 6 harboring *avrPphB* allele in pBBR1MCS2 broad-host range vector.

^d AvrPphB = wild-type protein, deletion or site directed mutants described in text.

^e Severity of HR was scored relative to rapid reaction. +++ = full HR; – = no response; ++ = intermediate reaction between – and +++.

^f Reaction was scored as +++ in cv. A43 leaves and ++ in pods.

1998). However, this mutant had an identical phenotype to NΔ63 in agroinfiltration tests on all resistant bean cultivars and in the nonhost species that were tested (Table 1). Although myristoylation of AvrPphB has not been demonstrated biochemically, it is tempting to speculate that an intact myristoylation motif may not be required for HR elicitation by AvrPphB or its truncated effectors in some plant hosts. An alternative scenario is that removal of the initiator methionine, a prerequisite for posttranslational myristoylation (Resh 1994), does not take place with the NΔ62 effector, as proposed for other Avr proteins (AvrB and AvrRpm1) that have an N-terminal myristoylation motif with the conserved glycine residue next to the initiator methionine. Regardless of which of these or other possible explanations is correct, our results suggest that the entire AvrPphB protein is required for full HR elicitation.

Differential phenotypes of site-directed AvrPphB mutants in resistant bean hosts and nonhost plants.

Since the G63 residue is part of both the putative myristoylation motif and the proteolytic cleavage site, we examined whether substitution of this residue affected host recognition of the protein or affected the processing or translocation, or both, in *Pseudomonas* strains by constructing two site-directed mutants. In one mutant, G63A, the glycine residue at position 63 was changed to alanine, a commonly used replacement that abolishes myristoylation (Resh 1999). In the other mutant, G63EC64F, the same residue was changed to glutamic acid and the cysteine at position 64 to phenylalanine, which is also excluded from the consensus myristoylation motif (Fig. 3). This mutant will be referred to hereafter as G63E for brevity.

We expressed these mutants in bean, tomato, tobacco, and *N. benthamiana* by the *Agrobacterium* transient expression assay. Surprisingly, both mutants induced cell death in the bean cvs. TG and A43, which carry the matching R3 resistance gene, as well as in tomato (Table 1, Fig. 2A and B). The observed cell death was phenotypically indistinguishable from that caused by wild-type protein. In contrast, no significant necrosis was observed in bean cvs. Canadian Wonder and A52, which lack the R3 gene, in tobacco, or in *N. benthamiana* (Table 1). These results suggest that the presence of the G63 residue is dispensable for full HR elicitor activity of AvrPphB in R3 bean cultivars and in tomato but is essential in tobacco and *N. benthamiana*, indicating that processing or myristoylation, or both, play an important role in *Nicotiana* species.

Because the G63A and G63E mutants were fully HR active in R3 bean cultivars when they were expressed via *Agrobacterium tumefaciens*, we examined whether they conferred avirulence to the virulent strain *P. syringae* pv. *phaseolicola* R6. Surprisingly, transconjugants expressing the mutant proteins did not elicit HR in pods or leaves of cv. TG (Table 1, Fig. 1) and developed water-soaked lesions, as did the virulent R6-EV transconjugants. One possible explanation is that the mutant proteins are either unstable in *Pseudomonas* strains, or they are not translocated via the type-III pathway. Next, we examined whether these proteins were stable and processed normally in R6-G63A and R6-G63E transconjugants. Western blot analysis showed both G63A and G63E accumulated to the same level as AvrPphB in the transconjugants but, unlike the wild-type protein, were not processed (Fig. 4). These results indicate that processing may be an essential step in type III-mediated deliv-

Fig. 2. A, *Agrobacterium*-mediated transient expression of *avrPphB* in resistant bean cultivar Tendergreen. Leaves were inoculated with suspension of *Agrobacterium tumefaciens* strain C58C1 carrying (1) vector pBin-Hyg-Tx, (2) pBin/*avrPphB*, (3) pBin/NΔ63, and (4) pBin/G63A. The leaf was photographed 3 days after inoculation. **B,** *Agrobacterium*-mediated transient expression of *avrPphB* in tomato cv. Rio Grande 76S. Leaves were inoculated as in A, and were photographed 3 days after inoculation. **C,** Necrotic phenotype of *Nicotiana benthamiana* inoculated with PVX::*avrPphB*, photographed 7 days after inoculation. Both species are nonhosts (i. e., resistant) to *Pseudomonas syringae* pv. *phaseolicola*.

ery. However, after inoculation of R6-G63A and R6-G63E transconjugants, cv. A43 developed a weak HR in pods (Fig. 1) and a reaction in leaves that was almost as rapid as that observed to wild-type AvrPphB. Taken together, the above results clearly indicate that the mutant proteins are potentially capable of translocation from bacteria to the plant; however, their delivery to the plant seems to be host dependent.

DISCUSSION

We have characterized the molecular signals of AvrPphB required for receptor recognition in various plant species and for type III-mediated delivery from *P. syringae* pv. *phaseolicola* to bean plants. We provide evidence that the N-terminal domain carries determinants whose alteration prevents effector processing in the bacterium, impairs its delivery to the plant cells via the bacterial TTSS in a plant-dependent manner, and results in differential HR phenotypes on different hosts. In particular, we show that the region defined by amino acid residue G63, which overlaps the putative myristoylation motif and borders the proteolytic cleavage site, is essential for HR-eliciting activity in *N. benthamiana* and tobacco plants but is dispensable in bean and tomato, suggesting that recognition specificity between AvrPphB and the *R* gene products is plant genotype dependent.

P. syringae pv. *phaseolicola* R6 expressing *avrPphB* was capable of delivering the AvrPphB signal to bean plants, and this process required a functional TTSS. Deletion of *hrpA*, the gene coding for the Hrp pilus structural protein, in *P. syringae* pv. *phaseolicola* R6 prevented the induction of HR in resistant bean cultivars. Furthermore, *P. syringae* pv. *phaseolicola* R6 transconjugants expressing N-terminal deletion mutants of AvrPphB did not cause HR in pods or leaves of the resistant bean cvs. TG and A43 carrying the matching *R3* gene. This was attributed primarily to a defect in translocation via the TTSS rather than to lack of effector recognition by the host, because the NΔ63 mutant effector triggered *R3* gene-specific necrosis in bean cultivars, although weaker than the wild-type effector, when the truncated genes were introduced into the plants through agroinfiltration.

The weaker HR phenotype of NΔ63 in agroinfiltrated bean leaves suggested that the N-terminal 63 residues of AvrPphB may carry some host-recognition determinants. An alternative explanation is that the truncated effector was less stable and accumulated to lower levels in bean. This possibility was investigated further by expressing the full-length and the truncated protein in three nonhost plants, namely tobacco, *N. benthamiana*, and tomato, by means of agroinfiltration and, in the latter two species, by PVX infection as well. In the agroinfiltration assay, this mutant gave weaker necrosis on all three nonhost species, similar to the results obtained in bean. In the PVX assay, the full-length AvrPphB effector gave progressive necrosis of the entire plant in *N. benthamiana* and in tomato, while the NΔ63 mutant caused similar necrosis in both species but with significant delay in comparison to the full-length protein. We could detect neither the full-length nor the mutant proteins (NΔ62, NΔ63, and NΔ109) in agroinfiltrated leaves or in PVX infected plants (only WT and NΔ63 were tested in this system) by Western blot analysis. Although the possibility that the weaker phenotype of the deletion mutants was a result of insufficient effector accumulation remains open, we consider it unlikely, at least in the case of PVX-mediated delivery of NΔ63, since this system gives very high levels of protein accumulation (Tampakaki and Panopoulos 2000).

Nimchuk and associates (2000) reported that disruption of the putative myristoylation motif of AvrPphB by a G63A substitution reduced the HR-eliciting activity and membrane localization in *Arabidopsis*. Although myristoylation of this effector

remains to be demonstrated biochemically, we hypothesized that the truncated NΔ63 effector may not be properly localized in planta, due to the absence of the G63 residue (presumed myristoyl group acceptor) and the consequent lack of myristoylation. This possibility was ruled out by the finding in the agroinfiltration assay that the NΔ62 mutant in which this residue was present next to an initiator methionine, a proper context for myristoylation (Resh et al. 1999), gave an identical phenotype to NΔ63. Taken together, the phenotypes observed with the deletion mutants indicate that the first 63 residues of AvrPphB are partially dispensable, while residues 64 to 109 are indispensable for effector recognition in *R3* of bean, tomato, tobacco, and *N. benthamiana*.

In planta expression of the two site-directed mutants, G63A and G63E, by the agroinfiltration assay revealed different phenotypes, depending on the host. Both mutants exhibited the same necrotic phenotype as the wild-type protein in resistant bean cultivars as well as in tomato, but neither of them elicited any necrosis in *N. benthamiana* or tobacco. These results were surprising and raised several interesting questions. First, what is the molecular and cellular basis of the differential reactions of these mutants in *N. benthamiana* and tobacco in comparison with the wild-type protein? Second, if myristoylation of AvrPphB occurs following proteolytic cleavage next to G63 and subsequent removal of the initiator methionine as hypothesized by Nimchuk and associates (2000), what functionally differentiates the G63A mutant from NΔ62? Clearly, the G63 residue is crucial for the HR elicitor activity of the full-length effector in the two *Nicotiana* species but makes no difference in bean and tomato and is immaterial in the case of the NΔ63 effector in all four species examined (Table 1). Unfortunately, we were not able to detect the wild-type or the mutant proteins in planta and thus cannot evaluate the relative importance of effector accumulation in planta processing and cellular localization in the differential effector-plant recognition. However, several other plausible scenarios are worthy of mention. First, not all proteins that contain a myristoylation motif are myristoylated in vivo, and some proteins that are myristoylated clearly do not require it for their function (Resh 1994; Ying-Tsu et al. 1998; Zhu et al. 1995). In a further variation of this theme, myristoylation may be important in some hosts but not in others. For example, it is possible that myristoylation of AvrPphB is not necessary in bean and tomato but is crucial in the two *Nicotiana* species. Second, it is conceivable that the G63A substitution does not alter the effector properties of the protein, including its potential myristoylation, in bean and tomato but does so in the two *Nicotiana* species (our results) and in *Arabidopsis* species (Nimchuk et al. 2000). Third, proteolytic processing (of the site-directed mutants) or removal of the initiator methionine (in the case of the NΔ62 mutant), both presumed prerequisites for myristoylation (see below), may not take place in the two *Nicotiana* species. If proteolytic processing occurred in these plants at the same site as in *E. coli*, we would expect the G63A protein to produce the same phenotype as NΔ62. In preliminary experiments, no degradation of ³⁵S-labeled AvrPphB protein was observed after incubation for 2 h with different plant extracts (data not shown). Finally, it is possible that the corresponding *R* receptors have different recognition specificities or that there are fundamental differences in where they localize in the cell and how they function in the different plants. Differential recognition of another Avr protein by cognate plant receptors found in different plants (tomato and tobacco) has been reported for AvrPto, suggesting that multiple *R* genes might have evolved different recognition specificities for the same Avr protein (Shan et al. 2000).

In addition to the differential phenotypes revealed by the agroinfiltration assay on the nonhost species, the site-directed

mutant effectors G63A and G63E gave differential HR responses on pods as well as on leaves of different *R3* bean cultivars upon delivery by *Pseudomonas* strains. Thus, both mutants gave typical HR on cv. A43 but not on cv. TG or on cv. Guatemala (data not shown), even though they were fully HR-active on both cultivars in the agroinfiltration assay. Clearly, the proteins are intrinsically capable of being translocated via the bacterial TTSS, since they did confer avirulence on cv. A43. The cultivar-specific differential phenotype of the site-directed mutant effectors could be rationalized in several ways. First, as argued in the case of the differential phenotypes on nonhost species, different bean cultivars may possess different *R3*-like alleles with slightly different specificities towards AvrPphB. This possibility finds some support in the finding that cv. A43 carries two *R3* homologues matching *avrPphB* but at different chromosomal locations, while cv. TG carries a single *R3* gene locus, as does cv. Guatemala (Teverson 1991). Second, besides differential specificities towards AvrPphB, the two *R3* gene homologues in cv. A43 may create a gene-dosage effect that could amplify HR signaling. Third, the receptor homologues in different cultivars may differ from each other in other respects (e. g., myristoylation or other types of acylation) connected with membrane localization. This possibility would be in line with hypotheses advanced by others (Nimchuk et al. 2000; Shan et al. 2000) that membrane-associated receptors might recruit nonmyristoylated effectors to an active complex or vice versa. A fourth possibility is that the host genotype may influence the translocation process itself. For example, cv. A43, but not cvs. TG or Guatemala, might trigger the TTSS-mediated effector delivery more efficiently. Such a hypothesis would not be unreasonable given the contact dependence of the TTSS-mediated effector established for other pathogens. Evaluation of these, and perhaps other, hypotheses must wait the cloning of *R3* gene homologues from bean and other species and the *in vivo* demonstration of AvrPphB myristoylation. Furthermore, in planta expression of the mutant effectors under regulated expression systems may enable the detection and quantification of the mutant proteins in planta and help clarify some of the questions raised in the present study.

MATERIAL AND METHODS

Bacterial strains and media.

Strains and transconjugants of *P. syringae* pv. *phaseolicola* and *P. fluorescens* were routinely grown on King's B medium (KB) at 28°C (King et al. 1954; Miller 1972). *E. coli* DH5a and *Agrobacterium tumefaciens* C58C1 containing the disarmed Ti helper plasmid pGV2260 (Deblaere et al. 1985) were grown using standard techniques (Sambrook et al. 1989). Antibiotics were usually used at the following concentrations

(µg/ml): ampicillin, 100; carbenicillin, 100; kanamycin, 50; rifampicin, 50.

Plasmid constructions.

Standard molecular biological procedures were performed as described by Sambrook and associates (1989), unless otherwise stated. The following plasmids were constructed for the present study. The DNA fragment encoding the full-length AvrPphB protein was isolated by polymerase chain reaction (PCR) amplification from a pBR322/*avrPphB* template and cloned in *E. coli* expression vector pT7-7 in the broad-host range vector pBBR1MCS2 (Kovach et al. 1995) for expression in *P. syringae* and in the viral expression vector pPVX202 (Sablowski et al. 1995) and the binary expression vector pBIN-Hyg-Tx (Gatz et al. 1992) for expression in plants. The primers AvrPphB-F and AvrPphB-R (5'-GTTTTGAGGTTTCATATGAAAATAGGTAC-3' and 5'-CGGGATCCGGCTGGGTTTTTGTCTTC-3', respectively) were designed to contain restriction sites (*Nde*I and *Bam*HI, underlined) to facilitate cloning in the corresponding sites of the pT7-7. A 50-µl PCR mixture containing 10 pmol of each primer, 100 ng of template 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.) was subjected to 25 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min extension at 72°C). The PCR product was digested with *Nde*I/*Bam*HI and cloned in corresponding sites of pT7-7. The resulting plasmid construct, pT7-7/*avrPphB*, was digested with *Nde*I/*Bam*HI and, after filling the ends with Klenow, ligated separately to the *Nru*I of pPVX202 and to the *Sma*I of pBIN-Hyg-Tx to give PVX202::*avrPphB* and pBIN/*avrPphB*, respectively. To construct the pBBR1MCS2/*avrPphB* plasmid, the coding region of *avrPphB* was obtained by PCR, using as template the pT7-7/*avrPphB* and the primers T7RBS (5'-GTTTAACGGTACCTTAAGAAGGAGATACATATG-3') and AvrPphB-R containing a *Kpn*I (underlined) and a *Bam*HI site, respectively. The forward primer, T7RBS, was designed to contain the ribosome binding site (RBS, bold) from pT7-7. The PCR was performed as above, except for the annealing temperature (42°C). The PCR product was digested with *Kpn*I and *Bam*HI and ligated to corresponding sites of pBBR1MCS2 vector.

To construct a deletion derivative of AvrPphB protein lacking amino acid residues 1 to 62, a PCR fragment encoding the carboxyl-terminal 210 amino acids of AvrPphB was amplified from the pBR322/*avrPphB* by using the forward primer Δ63-F (5'-CACTGGGCGATCATATGTGTGCATCCTC-3') and the reverse AvrPphB-R primer, simultaneously changing the glycine residue at position 63 to methionine (the ATG codon in the forward primer is italicised, *Nde*I underlined). The PCR product was digested with *Nde*I plus *Bam*HI and ligated to corresponding sites of pT7-7. The resulting pT7-7/Δ63 plasmid

AvrPphB: M-(X)₆₀-K↓GCASSSGVSLED...
 NΔ62: MGCASSSGVSLED...
 NΔ63: MCASSSGVSLED...
 G63A: M-(X)₆₀-KACASSSGVSLED...
 G63E: M-(X)₆₀-KEFASSSGVSLED...

Consensus: G-{EDRKHPFYW}-X2-[STAGCN]-{P}

Fig. 3. Comparison of AvrPphB myristoylation motif (in wild-type and mutant polypeptides, underlined) with a consensus myristoylation site derived from various proteins (Boutin 1997; Towler et al. 1988). The arrow indicates the cleavage site of AvrPphB as determined in *Escherichia coli* (Puri et al. 1997). Mutations in the myristoylation motif are indicated in bold.

Fig. 4. Immunoblot analysis of AvrPphB in total cellular lysates from *Pseudomonas syringae* pv. *phaseolicola* R6 transconjugants grown in rich media. Lane 1, *Pseudomonas syringae* pv. *phaseolicola* R6 expressing *avrPphB*; lane 2, *Pseudomonas syringae* pv. *phaseolicola* R6 expressing G63A; and lane 3, *Pseudomonas syringae* pv. *phaseolicola* R6 expressing G63E. Both wild-type and mutant genes were constitutively expressed from plasmid pBBR1MCS2.

was digested with *Nde*I and *Bam*HI and, after filling the ends with Klenow, ligated separately to the *Nru*I site of PVX202 and to the *Sma*I site of pBIN-Hyg-Tx to give PVX202::NΔ63 and pBIN/NΔ63 plasmids, respectively. Next, the portion of the coding region of *avrPphB* expressing the carboxyl-terminal 210 amino acids of AvrPphB was amplified from the pT7-7/NΔ63 by using the primers T7RBS and AvrPphB-R. The PCR product was digested with *Kpn*I/*Bam*HI and ligated to corresponding sites of pBBR1MCS2. Another deletion derivative of AvrPphB protein lacking amino-terminal amino acid residues 1 to 109 was constructed by PCR amplification. The fragment encoding the carboxyl-terminal 164 amino acids of AvrPphB was amplified from the pBR322/*avrPphB* by using the forward primer Δ109-F (5'-CGGAGTGGCTTCATATGA-GTAGTGACGGAG-3') and the reverse AvrPphB-R (the ATG codon in the forward primer is italicised, *Nde*I underlined). The PCR product was digested with *Nde*I/*Bam*HI and ligated in corresponding sites of pT7-7. The resulting pT7-7/NΔ109 plasmid was digested with *Nde*I and *Bam*HI and, after filling the ends with Klenow, ligated to *Sma*I of pBIN-Hyg-Tx to give pBIN/NΔ109. Next, the truncated coding region of *avrPphB* was amplified from the pT7-7/NΔ63, using primers T7RBS and AvrPphB-R. The PCR product was digested with *Kpn*I/*Bam*HI and ligated in corresponding sites of pBBR1MCS2 vector.

Site-directed mutagenesis of *AvrPphB*.

To generate the site-directed mutant G63A of AvrPphB, the following steps were followed. Plasmid pT7-7/*avrPphB* was used to mutagenize the glycine codon at position 63 to an alanine codon, according to the protocol described by Fisher and Pei (1997). The mutagenesis was carried out by PCR amplification using the primers 5'-GATAAAGCATGCGCATCC-TCTTCAGGC-3' (forward, mutagenic primer) and 5'-GCCC-AGTGTCAATTGCCAGGAAT-3' (divergent, nonoverlapping primer). The mutagenic primer contains the mutant codon for alanine (italicised) and an *Sph*I restriction site (underlined).

Double mutant G63EC64F was obtained by PCR amplification as above, except for using the forward primer 5'-GATAAA-GAATTCGCGCATCCTCTTCAGGC-3'. The mutagenic primer contains two mutant codons, replacing the glycine and cysteine codons at positions 63 and 64 of *avrPphB* with a glutamic acid and phenylalanine codons (italics), respectively, while simultaneously generating an *Eco*RI restriction site (underlined). Both mutations were confirmed by restriction digestion and sequencing. For expression in *Pseudomonas* strains and in planta, both mutations were introduced in pBBR1MCS2 and pBIN-Hyg-Tx, respectively, by the same procedure described above.

Triparental mating.

Introduction of the binary plasmid constructs into *A. tumefaciens* C58C1 (pGV2260) was carried out via triparental mating (Rogers et al. 1988), and transconjugants were selected on Luria-Bertani medium (Sambrook et al. 1989) 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 broad-host range plasmid constructs were introduced into rifampicin-resistant *P. syringae* pv. *phaseolicola* race 6 and the *P. fluorescens* strain as above. Transconjugants were selected in KB medium containing the appropriate antibiotics (80 µg of rifampicin per ml, 50 µg of kanamycin per ml, 12 µg of tetracycline per ml). Both *Agrobacterium* and *Pseudomonas* transconjugants were confirmed by PCR analysis.

Plants and pathogenicity tests.

HR assays or pathogenicity tests, or both, were carried out on species *N. benthamiana*, *N. tabacum* cv. Xanthi and cv. Wis-

consin 38, *L. esculentum* cv. Rio Grande (near isogenic lines 76S and 76R) (all nonhosts to *P. syringae* pv. *phaseolicola*) and on the French bean *Phaseolus vulgaris* cvs. TG, A43, Canadian Wonder, and A52. The genotypes of the bean cultivars regarding the presence or absence of the matching R3 gene are given in Table 1. Bean pods were inoculated as previously described (Harper et al. 1987; Hitchin et al. 1989). The compatible interaction on pods is expressed in the form of water-soaked lesions, while incompatibility is in the form of sunken brown lesions (HR) at the sites of injection within 2 days of incubation at 25°C. Bean primary leaves were inoculated by injection of bacterial suspensions at a concentration of 2×10^6 CFU ml⁻¹ to 2×10^8 CFU ml⁻¹ in 10 mM MgCl₂ using a needleless syringe and then incubated at 25°C. Compatible interactions (water-soaking) were recorded after 3 to 4 days and incompatible reactions after 24 h.

Agrobacterium transient expression assay.

The transient expression of the *avrPphB* gene in *N. tabacum* cv. Xanthi, *N. benthamiana*, *L. esculentum* cv. Rio Grande near isogenic lines 76R and 76S, and *Phaseolus vulgaris* plants was done according to the method described by Kapila and associates (1997). Briefly, *A. tumefaciens* strain C58C1 containing the Ti-plasmid pGV2260 (Deblaere et al. 1985) or individual transconjugants were grown in 5 ml yeast extract beef peptone (YEB) medium (Kapila et al. 1997) containing the appropriate antibiotics. Following overnight growth at 28°C, bacteria were pelleted by centrifugation and resuspended in 5 ml YEB medium containing 10 mM N-morpholino-ethanesulfonic acid (MES) (pH 5.6), antibiotics, and 20 µM acetosyringone. The culture was grown overnight to log phase (OD₆₀₀ = 0.5 to 0.8) at 28°C, centrifuged, and resuspended in MMA medium (Murashige-Skoog salts, 10 mM MES, 20 g of sucrose per l, pH 5.6, and 200 µM acetosyringone) to a final OD₆₀₀ of 2.4. The *Agrobacterium* cell suspension was kept at 22°C for 1 h. Bacterial suspensions were infiltrated into fully expanded primary leaves of plants using a needleless syringe.

PVX inoculations.

Four-week-old *N. benthamiana* plants were inoculated with the viral expression vector PVX202, PVX202::*avrPphB*, or PVX202::NΔ63 by gently rubbing leaves dusted with carborundum, according to Sablowski and associates (1995). Two leaves of each plant were inoculated with 5 to 10 µg Qiagen-purified DNA dissolved in 30 µl of 50 mM sodium phosphate buffer, pH 7.0.

P. syringae protein extraction and immunoblot analysis.

Overnight *P. syringae* pv. *phaseolicola* cultures in KB with appropriate antibiotics (1 ml) were pelleted, washed with 10 mM MgCl₂, and resuspended in 250 µl Laemmli buffer. Samples were boiled for 5 min at 100°C, and 20 µl were loaded on 14% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membrane, using standard procedures. Immunoblotting was carried out using antisera raised in rabbits against AvrPphB (Puri et al. 1997) at a 1:5000 dilution. The membranes were developed with a horseradish peroxidase-conjugated anti-rabbit IgG antibody using chemiluminescent substrates (Pierce, Rockford, IL, U.S.A.).

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