

RESEARCH ARTICLE

Expression of the *Pseudomonas syringae* Avirulence Protein AvrB in Plant Cells Alleviates Its Dependence on the Hypersensitive Response and Pathogenicity (Hrp) Secretion System in Eliciting Genotype-Specific Hypersensitive Cell Death

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The nonpathogenic bacteria *Pseudomonas fluorescens* and *Escherichia coli* can elicit a genotype-specific hypersensitive response (HR) in plants if they express both the HR and pathogenesis (Hrp) protein secretion system and the HrpZ harpin from *P. syringae* pv *syringae* 61 and a *P. syringae* avirulence (*avr*) gene whose presence is recognized by a corresponding disease resistance gene in the plant. We have found that the recognition event appears to require transfer of the Avr protein into the plant cell. Elicitation of a genotype-specific HR was observed with *avrB*⁺ *P. fluorescens* in soybean and Arabidopsis plants carrying resistance genes *RPG1* and *RPM1*, respectively, and with *avrPto*⁺ *E. coli* in tomato plants carrying resistance gene *PTo*, but only if the Hrp secretion system, HrpZ, and the appropriate Avr proteins were produced in the same bacterial cell. The failure of *avrB* hyperexpression and exogenous AvrB or HrpZ to alleviate these requirements in soybean and Arabidopsis suggests that the site of AvrB action is not in the bacterial cell or plant apoplast. An Arabidopsis *rps3* (*rpm1*) *glabrous1* mutant was transformed with constructs expressing *avrB* and was crossed with an Arabidopsis ecotype Columbia (*RPM1* *GLABROUS1*) plant. F₁ seedlings (identified by their kanamycin-resistant, pubescent phenotype) exhibited extensive necrosis on cotyledon leaves 10 days postgermination. Ecotype Columbia and *rps3-1* leaves biolistically cobombarded with plasmids expressing the β -glucuronidase (*GUS*) gene and *avrB* failed to produce *GUS* activity (indicative of cell death) only when *RPM1* and *avrB* were present in the leaf. Thus, both stable and transient expression of *avrB* in Arabidopsis resulted in *RPM1*-dependent necrosis, and the only demonstrable site of action for AvrB was inside plant cells.

INTRODUCTION

Pseudomonas syringae is a host-specific plant pathogen whose individual strains are assigned to pathovars and races on the basis of their pathogenic interactions with plants. Compatible interactions with a host plant are accompanied by prolonged bacterial multiplication and the progressive development of disease symptoms over many days. In contrast, the incompatible interactions of a pathovar with nonhost plant species, or of races within a pathovar with resistant cultivars of its host, are marked by the development of the hypersensitive response (HR). The HR is characterized by rapid, localized death of plant cells at the site of pathogen invasion and is associated with plant defense (Goodman and Novacky, 1994). The ability of

P. syringae and many other Gram-negative plant pathogens to elicit the HR, multiply, or cause disease in plants is controlled by HR and pathogenicity (*hrp*) genes (Bonas, 1994), and host specificity at the race-cultivar level is determined by avirulence (*avr*) genes (Keen, 1990; Dangl, 1994).

Numerous bacterial *avr* genes have been cloned on the basis of their ability to trigger the HR when heterologously expressed in a pathogen that is normally compatible with a series of test cultivars of the host (Keen, 1990). Although *avr* genes are named by their dominant phenotype in such incompatible interactions, several contribute to virulence in compatible interactions, and their coregulation with the *hrp* genes in *P. syringae* suggests an underlying role in Hrp-mediated pathogenesis (Huynh et al., 1989; Dangl, 1994). The *hrp* genes are required for pathogenic bacteria to express *avr*

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gene phenotypes, and many encode components of a type III protein secretion system conserved in both plant and animal pathogens (Van Gijsegem et al., 1993; Dangl, 1994). The HrpZ harpin is one protein known to be secreted by the *P. syringae* Hrp system, and it is capable of eliciting hypersensitive necrosis when infiltrated into the leaf intercellular spaces of tobacco and several other plants (He et al., 1993). The site of action of AvrB and other Avr proteins is less clear: there are no reports of Avr proteins having elicitor activity when infiltrated into leaves, and these proteins have been observed only in the bacterial cytoplasm, even in infected plants, whereas corresponding resistance gene products are predicted to be in the plant cytoplasm (Brown et al., 1993; Dangl, 1994; Young et al., 1994; Staskawicz et al., 1995). The capacity of the type III protein secretion systems of *Yersinia*, *Shigella*, and *Salmonella* spp to secrete some proteins into the medium and others directly into host cells, during transient transfer events triggered by host cell contact, raises the possibility that the homologous pathway in *P. syringae* and other plant pathogens may function similarly with HrpZ and Avr proteins, respectively (Rosqvist et al., 1994, 1995; Sory and Cornelis, 1994).

Cosmid pHIR11 carries a 25-kb cluster of *hrp* genes, cloned from *P. syringae* pv *syringae* 61, which enables saprophytic bacteria like *Escherichia coli* and *P. fluorescens* to secrete HrpZ to the bacterial milieu in culture and to elicit the HR in tobacco leaves (Huang et al., 1988; Alfano et al., 1996). The *hrpZ* gene is located in the pHIR11 *hrp* cluster in an operon that also encodes components of the Hrp secretion pathway (Preston et al., 1995). A functionally nonpolar Δ *hrpZ*::neomycin phosphotransferase II (*nptII*) mutation abolished the ability of *E. coli* MC4100 carrying pHIR11 to elicit a typical HR, indicating that HrpZ is important to the ability of the pHIR11 *hrp* cluster to confer elicitor activity to saprophytic bacteria (Alfano et al., 1996). However, neither purified HrpZ nor *P. fluorescens* carrying pHIR11 elicits the HR in soybean, suggesting that the incompatible *P. syringae* strains capable of doing so must produce at least one factor in addition to HrpZ and the Hrp secretion components, both of which appear to be generally conserved in sequence and function irrespective of host range in *P. syringae* (Preston et al., 1995).

One candidate factor, the *P. syringae* pv *glycinea* *avrB* gene, was previously cloned by its ability to confer HR-eliciting activity to normally compatible *P. s. glycinea* races in soybean cultivars harboring the corresponding disease resistance gene, *RPG1* (Staskawicz et al., 1987). Subsequently, *avrB* was shown to function in a heterologous pathosystem involving *P. syringae* pv *tomato* and *Arabidopsis* (Innes et al., 1993). *Arabidopsis rps3* mutants defective in the perception of the AvrB signal have been identified (Bisgrove et al., 1994), and all *rps3* mutations have been mapped to a disease resistance gene, *RPM1*, which is involved in the perception of the signal from a structurally different *avr* gene, *avrRpm1*. The cloned *RPM1* gene, when introduced into *rps3* and *rpm1* mutants, confers resistance to virulent bacteria carrying either *avrRpm1* or *avrB* (Grant et al., 1995).

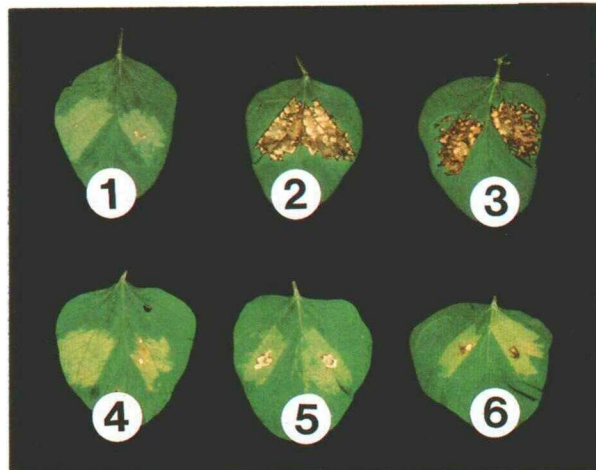


Figure 1. Elicitation of a Genotype-Specific HR in Soybean by *P. fluorescens* Carrying pHIR11 and Expressing *avrB*.

Soybean cultivars Harosoy (*RPG1*) (leaves 1 through 3) and Acme (*rpm1*) (leaves 4 through 6) were infiltrated with bacteria at a concentration of 5×10^8 cells per mL and then photographed 7 days later when a nonspecific chlorosis (which typically develops at this time after infiltration with very high levels of saprophytic bacteria) marked the infiltrated areas. Complete collapse, typical of the HR, occurred within 48 hr in leaves 2 and 3. Leaves 1 and 4 were inoculated with *P. fluorescens* carrying pHIR11; leaves 2 and 5 were inoculated with *P. fluorescens* carrying pHIR11 and pAVRB1; and leaves 3 and 6 were inoculated with *P. fluorescens* carrying pHIR11 and pAVRB-FLAG1.

It has recently been found that saprophytic bacteria carrying pHIR11 are capable of eliciting a genotype-specific HR (Collmer et al., 1996; Hutcherson et al., 1996). In this study, we used *P. fluorescens* carrying pHIR11, *hrp* mutant derivatives of pHIR11, and FLAG epitope-tagged AvrB to explore the requirements for delivery of the AvrB signal, and we used assays involving stable and transient expression of *avrB* in *Arabidopsis* plants differential for *RPM1* to explore the site of AvrB action. We also showed, with *E. coli* MC4100 carrying pHIR11 and the *avrPto* avirulence gene, that the observed requirements for Avr signal delivery are not limited to *P. fluorescens* and AvrB. The experiments suggest that at least some Avr proteins act within plant cells after delivery by the Hrp secretion system.

RESULTS

P. fluorescens 55 Carrying pHIR11 Elicits an *avrB*-*RPG1*-Dependent HR in Soybean

We first constructed pAVRB-FLAG1, which produces AvrB with an eight-amino acid C-terminal FLAG epitope fusion, so that

we could monitor the expression and distribution of AvrB. pAVRB1 (Tamaki et al., 1988) and pAVRB-FLAG1 were equally effective in causing *P. s. glycinea* race 4, at a concentration of 5×10^8 cells per mL, to elicit a typical HR within 48 hr in *RPG1* soybean cultivars Harosoy and Norchief, but not in *rpm1* cultivars Acme and Centennial (data not shown). Thus, AvrB-FLAG retained its function as an *RPG1*-specific Avr protein.

To determine whether expression of *avrB* could enable *P. fluorescens* carrying pHIR11 to elicit a genotype-specific HR in soybean and Arabidopsis, we transformed the bacterium with pAVRB1 and pAVRB-FLAG1. The transformants then elicited the HR in cultivar Harosoy but not in cultivar Acme (Figure 1), in Norchief but not in Centennial, and in Arabidopsis ecotype Columbia (Col; *RPM1*) but not in its *rps3* (*rpm1*) derivative (Figure 2). Thus, pHIR11 carries the necessary genes for a saprophytic relative of *P. syringae* to deliver the AvrB signal to diverse plants.

Elicitation of a Genotype-Specific HR in Soybean and Arabidopsis by *avrB*⁺ *P. fluorescens* 55 Carrying pHIR11 Cells Is Dependent on Both HrpZ and the Hrp Secretion System

We explored the requirements for pHIR11-mediated delivery of the AvrB signal with pCPP2089, an existing *hrcC::TnphoA* (*hrcC* was previously designated *hrpH* [Bogdanove et al., 1996]) secretion pathway mutant (Huang et al., 1991), and with pCPP2274, a newly constructed unmarked Δ *hrpZ* pHIR11 mutant. pCPP2274 was made by precisely deleting the *hrpZ* open reading frame (ORF) from a *hrcJ-hrpBZASR*⁺ (*hrcJ* was previously designated *hrpC* [Bogdanove et al., 1996]) plasmid and then introducing the deletion into pHIR11 by homologous recombination of flanking sequences. When infiltrated into leaves of soybean plants harboring *RPG1* (Harosoy and Norchief) at a concentration of 5×10^8 cells per mL,

Bacteria or protein infiltrated	Schematic of Hrp/HrpZ/AvrB phenotype	AvrB in inoculated soybean leaves	HR elicitation in			
			Arabidopsis <i>RPM1</i>	Arabidopsis <i>rpm1</i>	Soybean <i>RPG1</i>	Soybean <i>rpm1</i>
Pf(pHIR11)	(a)	ND	-	-	-	-
Pf(pHIR11, pAVRB1)	(b)	ND	+	-	+	-
Pf(pHIR11, pAVRB-FLAG1)	(c)		+	-	+	-
Pf(pHIR11, pAVRB-FLAG2)	(d)		+	-	+	-
Pf(pCPP2274, pAVRB-FLAG2)	(e)	ND	-	-	-	-
Pf(pCPP2089, pAVRB-FLAG2)	(f)		-	-	-	-
Pf(pCPP2274, pAVRB-FLAG2) + Pf(pHIR11)	(g)		-	-	-	-
Pf(pCPP2274, pAVRB-FLAG2) + HrpZ	(h)	ND	-	-	-	-
AvrB-FLAG	(i)	ND	-	-	-	-

Figure 2. Elicitation of Genotype-Specific Necrosis in Leaves of Arabidopsis and Soybean Plants Carrying *RPM1* and *RPG1*, Respectively, by *P. fluorescens* Cells Differentially Expressing Components of the Hrp Secretion System and *avrB*.

The cosmids or plasmids carried in *P. fluorescens* (Pf) are given in parentheses. The band in row i is from a Coomassie blue-stained gel of the purified AvrB-FLAG used in the bioassay. The AvrB bands in the third column are from immunoblots of protein recovered from soybean Harosoy leaves inoculated 1 hr earlier with Hrp-derepressed cultures of the indicated bacteria at 10^9 cells per mL (ND, not done). The amount of AvrB-FLAG in row c is undetectable at this exposure, and replicate assays revealed no reproducible difference between rows d, f, and g. HR elicitation was scored 48 hr after inoculating leaves with indicated bacteria at 5×10^8 cells per mL. Soybean tests were performed with *RPG1* cultivars Harosoy and Norchief and *rpm1* cultivars Acme and Centennial. (+) indicates HR; (-) indicates no response. Arrow, functioning (*hrcC*⁺) Hrp secretion pathway; z, residual HrpZ left in a *hrpZ*⁺ cell that is secreting HrpZ; B, *avrB*⁺ cell; **B**, cell hyperexpressing *avrB*⁺; B?, the small amount of AvrB that is postulated to be transferred to plant cells.

P. fluorescens carrying pCPP2089 and pAVRB-FLAG1 and *P. fluorescens* carrying pCPP2274 and pAVRB-FLAG1 failed to elicit the HR (data not shown).

To explore further the involvement of HrpZ and the Hrp secretion system in the delivery of the AvrB signal, we prepared purified AvrB-FLAG and HrpZ, and we constructed pAVRB-FLAG2, in which the *nptII* promoter drives the production of ~100-fold more AvrB-FLAG than is produced from the *lac* promoter in pAVRB-FLAG1 when in *P. fluorescens*. We confirmed that pAVRB-FLAG2 was able to convert *P. s. glycinea* race 4 to incompatibility on *RPG1* cultivars (data not shown). Immunoblot analysis (Figure 2, third column) confirmed that *P. fluorescens* produces much higher levels of AvrB-FLAG in soybean leaves when carrying pAVRB-FLAG2.

As depicted in Figure 2, a genotype-specific HR was produced only when HrpZ and AvrB were produced in the same secretion-competent *P. fluorescens* cell (rows b to d). This requirement could not be alleviated by the production of large excesses of AvrB (rows e to h), by coinoculation of secretion-competent bacteria separately producing HrpZ and AvrB (row g), by supplying purified exogenous HrpZ at a level 10-fold higher than that required to elicit the HR in tobacco (row h), or by infiltrating purified AvrB into leaves at a level 1000-fold higher than that required for bacteria to deliver the AvrB signal (row i).

***avrPto*⁺ *E. coli* MC4100 Carrying pHIR11 Elicits a PTO-Dependent HR in Tomato**

To determine whether the requirements for pHIR11-mediated delivery of the AvrB signal are unique to *P. fluorescens* 55 and AvrB or are more general, we transformed *E. coli* MC4100 carrying pHIR11 with pPtE6, which carries *avrPto* from *P. s. tomato* (Ronald et al., 1992). HrpZ and *P. fluorescens* 55 carrying pHIR11 are capable of eliciting an HR in tomato (Preston et al., 1995); however, apparently due to weaker *hrp* expression, *E. coli* MC4100 carrying pHIR11 elicits at most a very weak HR in tomato. *E. coli* MC4100 carrying pHIR11 and pPtE6 reproducibly produced a typical hypersensitive collapse in the tomato Rio Grande isolate carrying *PTO*, whereas only an occasional or spotty necrosis was observed if the isolate lacked *PTO* or the bacteria lacked *avrPto* or *hrpZ* (data not shown). In additional experiments, *E. coli* MC4100 carrying pCPP2089 and pPtE6 failed to elicit any necrosis in tomato, and the use of mixed bacterial genotypes, analogous to the Figure 2 (row g) experiment, did not restore HR activity (data not shown). Thus, saprophytic bacteria can elicit genotype-specific hypersensitive necrosis in the absence of all *P. syringae* genes other than those in the *hrp* cluster, but effective heterologous delivery of *avr* signals requires both HrpZ and a functional Hrp secretion system.

Stable Expression of *avrB* Is Lethal in Transgenic Arabidopsis Plants Carrying *RPM1*

To test directly the hypothesis that AvrB acts within plant cells rather than in bacterial cells or the plant apoplast, we transformed the Arabidopsis *rps3-1* mutant with *avrB*. The coding region of *avrB* was amplified from pAVRB1 (Tamaki et al., 1988) by polymerase chain reaction (PCR), and it was cloned into plant transformation vector pKYLX71::35S² (Maiti et al., 1993), with or without the signal peptide (sp) sequence of the tobacco pathogenesis-related protein PR-1b (Cornelissen et al., 1987). The tobacco PR-1b signal peptide sequence has been used to secrete foreign proteins to the plant apoplast (Lund and Dunsmuir, 1992). Therefore, the *sp-avrB* product would not be expected to accumulate at substantial levels in the plant cell, whereas AvrB without the signal peptide would. pKYLX71::35S²/*avrB* and pKYLX71::35S²/*sp-avrB* were introduced into Arabidopsis Col *glabrous1* (*gl1*) and *rps3-1 gl1* plants via Agrobacterium-mediated transformation. The Arabidopsis *gl1* morphological mutant, which does not produce trichomes (Marks and Feldmann, 1989), exhibits a typical HR following infiltration with incompatible *P. syringae* strains (data not shown), and except where noted, it was used throughout this work. Transformants were selected on plant nutrient medium (Bechtold et al., 1993) supplemented with 50 µg/mL kanamycin. In two independent transformations, only *rps3-1* plants harboring the *sp-avrB* sequence were obtained. No kanamycin-resistant, true leaf-bearing plants were observed in selection plates intended to produce the following transformants: Col plants expressing *sp-avrB* or *avrB* and *rps3-1* plants expressing *avrB*. The surprising failure to recover transformants when *avrB* was transformed into *rps3-1* Arabidopsis plants likely resulted from plant lethality due to high levels of AvrB inside plant cells.

rps3-1 plants are fully susceptible to infection by virulent bacteria carrying *avrRpm1* or *avrB* due to a mutation in the *RPM1* plant resistance gene (Bisgrove et al., 1994). No HR was expected in this line when it was inoculated with bacteria carrying *avrRpm1* or *avrB*. Surprisingly, most *rps3-1* plants harboring the *sp-avrB* sequence showed varying degrees of tissue necrosis resembling HR lesions (e.g., transformant line 22 in Figure 3C). The necrotic symptom was a stable phenotype; T₂ plants showed the same degrees of necrosis as their respective T₁ parental plants (data not shown). Only line 12 did not show any necrotic symptoms (Figure 3B). The degree of necrotic symptoms on leaves of *rps3-1* (*sp-avrB*) plants was correlated with the level of expression of the *sp-avrB* sequence. The *sp-avrB* transcript level was higher in line 22 than in line 12 (Figure 3E). As expected, no *avrB* transcript was detected in untransformed *rps3-1* plants (Figure 3E). The residual HR phenotype exhibited by many *rps3-1* plants expressing *avrB* suggests that the *rpm1* mutation in *rps3-1* is not null, which is consistent with the possible truncation of its product at amino acid 818 (Grant et al., 1995), or that *avrB* interacts with a second weak plant resistance gene.

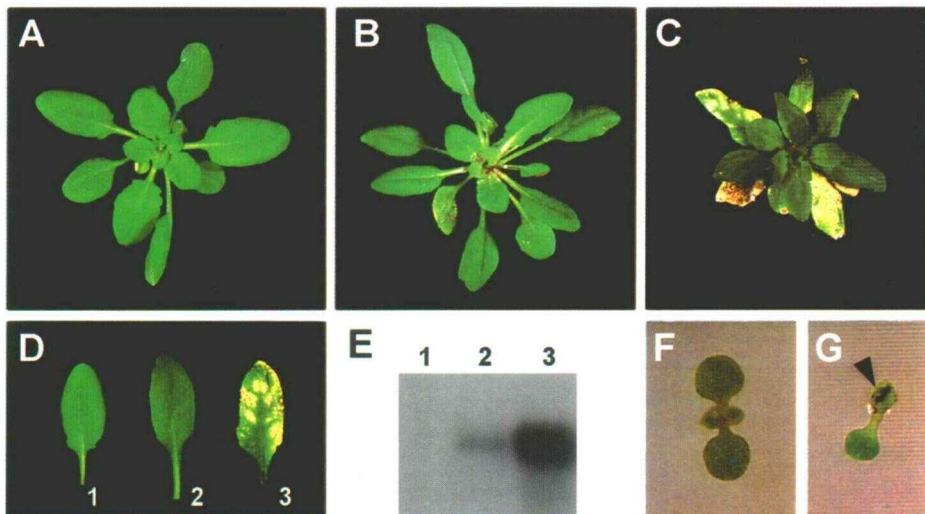


Figure 3. Stable Expression of *avrB* in Arabidopsis Plants and Necrosis in F₁ Seedlings Expressing Both *avrB* and *RPM1*.

(A) to (C) Five-week-old *rps3-1*, *rps3-1 (sp-avrB)* line 12, and *rps3-1 (sp-avrB)* line 22 plants germinated in plant nutrient agar plates and transferred to soil 1 week later.

(D) Representative leaves from *rps3-1* (leaf 1), *rps3-1 (sp-avrB)* line 12 (leaf 2), and *rps3-1 (sp-avrB)* line 22 (leaf 3).

(E) RNA gel blot analysis of the *avrB* transcript in the leaf tissue of *rps3-1* (lane 1), *rps3-1 (sp-avrB)* line 12 (lane 2), and *rps3-1 (sp-avrB)* line 22 (lane 3).

(F) Ten-day-old seedling of *rps3-1 (sp-avrB)* line 12 grown on plant nutrient agar showing no HR lesions.

(G) Ten-day-old F₁ progeny seedling of Col (*GL1*) × *rps3-1 (sp-avrB)* line 12 grown on plant nutrient agar showing dark necrotic HR lesions that are indicated by an arrowhead on a cotyledon leaf.

We chose *rps3-1 (sp-avrB)* line 12 for further analysis of *RPM1*-dependent HR initiation by AvrB. Reciprocal genetic crosses were performed between *rps3-1 (sp-avrB)* line 12 and Col plants. The development of F₁ seedlings was monitored for 2 weeks postgermination in plant nutrient plates supplemented with kanamycin. Although kanamycin-resistant seedling progeny of *rps3-1 (sp-avrB)* line 12 grew normally and gave rise to true leaves without necrotic symptoms (Figure 3F), the kanamycin-resistant, pubescent F₁ seedlings of Col *GL1* × *rps3-1 (sp-avrB)* line 12 exhibited extensive necrosis (dark lesions) on cotyledon leaves 10 days postgermination (Figure 3G). No true leaves were formed, and seedlings died shortly thereafter. Similar crosses were performed between *rps3-1 (sp-avrB)* line 34 (exhibiting mild necrosis) and Col. The F₁ progenies of this cross also exhibited extensive necrosis on cotyledon leaves, and seedlings died within 2 weeks postgermination (data not shown). Because *rps3-1 (sp-avrB)* line 12 expressed a very low level of the *sp-avrB* transcript, the lethality of the F₁ seedling of the Col × *rps3-1 (sp-avrB)* line 12 suggests that very low levels of AvrB, transiently present in the cytoplasm, are sufficient to cause an HR in Col plants containing the *RPM1* gene. This observation is consistent with our inability to obtain any transgenic Col plants expressing *avrB* or *sp-avrB*.

Transient Expression of *avrB* in Arabidopsis Plants Carrying *RPM1* Is Lethal

Because no transgenic plants expressing AvrB without the PR-1b signal peptide were obtained in our experiments, we determined the activity of AvrB without the PR-1b signal peptide by using a transient expression protocol similar to the one reported by Mindrinos et al. (1994). This biolistic transient expression system is based on the prediction that genotype-specific hypersensitive cell death precludes accumulation of the product of a β -glucuronidase (*GUS*) reporter, as indicated by reduced *GUS* activity in cells cointroduced with genes encoding *GUS* and an elicitor of the HR. We bombarded Col and *rps3-1* leaves with pKYLX71::35S²/*GUS* plus pKYLX71::35S² (as a control) or with pKYLX71::35S²/*GUS* plus pKYLX71::35S²/*avrB*. As shown in Figure 4, cells in Col and *rps3-1* leaves bombarded with pKYLX71::35S²/*GUS* plus pKYLX71::35S² expressed high levels of *GUS* activity. These are indicated by large blue spots. Cells in Col leaves bombarded with pKYLX71::35S²/*GUS* plus pKYLX71::35S²/*avrB* consistently showed no or very low levels of *GUS* activity. In contrast, high levels of *GUS* activity were observed in *rps3-1* leaves bombarded with pKYLX71::35S²/*GUS* plus pKYLX71::35S²/*avrB*, again demonstrating that the AvrB-triggered HR cell death is

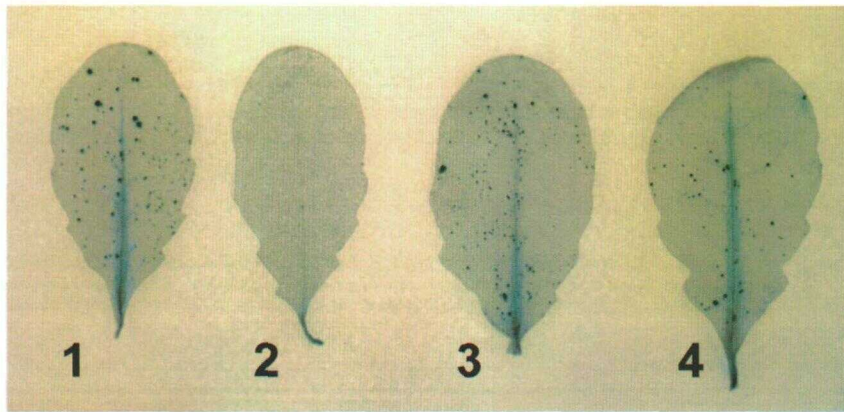


Figure 4. Effects on GUS Activity of Transient Expression of *avrB* in Arabidopsis Ecotype Col and *rps3-1* (*rpm1*) Leaves.

Leaves 1 and 2 are from 5-week-old Col plants, and leaves 3 and 4 are from 5-week-old *rps3* plants. Leaves were cobombarded with either pKYLX71::35S² (control vector) plus pKYLX71::35S²/*GUS* (leaves 1 and 3) or pKYLX71::35S²/*avrB* plus pKYLX71::35S²/*GUS* (leaves 2 and 4). The leaves were photographed after being incubated for 24 hr and then histochemically assayed for GUS activity.

dependent on the *RPM1* gene. In most experiments, *rps3-1* leaves showed a marginal but reproducible reduction of large blue spots when bombarded with pKYLX71::35S²/*GUS* plus pKYLX71::35S²/*avrB*, compared with those bombarded with pKYLX71::35S²/*GUS* plus pKYLX71::35S². This is consistent with the residual HR-eliciting activity of *AvrB* observed in most *rps3-1* (*sp-avrB*) plants (Figures 3A to 3D).

DISCUSSION

We propose that some *Avr* proteins are dependent on HrpZ and the Hrp secretion system for delivery into plant cells, where they either promote parasitism or elicit a genotype-specific HR if the corresponding resistance gene is present. This hypothesis is founded on indirect but compelling evidence that *AvrB* delivered by *P. fluorescens* carrying pHIR11 and *AvrPto* delivered by *E. coli* MC4100 carrying pHIR11 function only in the plant cell and on direct evidence that *AvrB*, when expressed in plant cells, elicits genotype-specific necrosis. Here, we discuss the evidence for Hrp-dependent delivery of *Avr* proteins into plant cells and the implications of such a delivery system for bacterial pathogenesis.

There are three hypotheses providing alternatives to the one in which *Avr* proteins are delivered in a Hrp-dependent manner into plant cells. They are consistent with the observed requirement of HrpZ and the Hrp secretion system for the genotype-specific HR. (1) *AvrB* and *AvrPto* may resemble *P. s. tomato* *AvrD* in directing the bacterial synthesis of low molecular weight elicitors of genotype-specific HR (Kobayashi et al., 1990), and these signals (unlike the syringolide products of *AvrD*) may be insufficient to elicit the HR in appropriate plant cells without an independent second signal from HrpZ. (2) *AvrB*

and *AvrPto* may be secreted into the apoplast along with HrpZ, and the synergistic action of the secreted proteins from the two classes may lead to a genotype-specific HR. (3) *AvrB* and *AvrPto* may modify HrpZ such that it becomes a genotype-specific elicitor of the HR.

The experiments summarized in Figure 2 provide strong evidence against the first two alternative hypotheses. The immunoblot assays confirmed that levels of *AvrB* much higher than those required to elicit the HR (row c) are present in interactions that do not result in the HR because of *hrcC* (row f) or *hrpZ* (rows e and g) mutations. Furthermore, when HrpZ is supplied exogenously by *P. fluorescens* carrying pHIR11 (row g) or as purified protein at a concentration 10-fold higher than that required to elicit the HR in tobacco (row h), *P. fluorescens* cells hyperexpressing *avrB* still failed to elicit the HR. If *AvrB* acted indirectly by producing a low molecular weight elicitor in bacterial cells or if *AvrB* and HrpZ acted synergistically in the apoplast to elicit the HR, then an HR should have been observed in rows g and h.

Evidence against the third alternative hypothesis follows from the observation that isoelectric focusing, SDS-denatured, and native polyacrylamide gels revealed no differences in the pl and molecular weight of HrpZ in the concentrated supernatants of cultures of *P. fluorescens* carrying pHIR11 with or without pAVRB-FLAG2 (data not shown). Similarly, the identical spectrum of activity in different plants of HrpZ purified from *P. s. tomato* or from *E. coli* expressing *hrpZ*_{Pst} (Preston et al., 1995) diminishes the likelihood of such a modification. Finally, it is worth recalling that purified *AvrB*-FLAG, at a concentration 1000-fold higher than that required by *P. fluorescens* carrying pHIR11 and pAVRB-FLAG1 to elicit the HR, was ineffective when infiltrated into soybean leaves. Thus, our observations argue by default against *AvrB* action in any location other than the interior of plant cells.

Relevant precedence for the transfer of bacterial virulence proteins into living host cells is found with the VirD2 and VirE2 proteins of *Agrobacterium* (Zupan and Zambryski, 1995) and the type III protein secretion systems of animal pathogenic *Yersinia*, *Shigella*, and *Salmonella* spp (Rosqvist et al., 1994, 1995). Transfer of the T-DNA nucleoprotein complex into plant cells demonstrates that the plant cell wall is not an impenetrable barrier to specialized bacterial protein secretion systems. The extensive homologies between the type III protein secretion components of *P. s. syringae* 61 and those of animal pathogens like *Y. enterocolitica* (and also components of the bacterial flagellar biogenesis apparatus) suggest that the Hrp secretion system may similarly permit the post-translationally regulated, polarized transfer of proteins into the host via a translocation apparatus involving cytoplasmic, inner membrane, outer membrane, and extracellular proteins (Michiels et al., 1991; Huang et al., 1992, 1993, 1995; Wattiau and Cornelis, 1993; Lidell and Hutcheson, 1994; Rosqvist et al., 1994, 1995; Sory and Cornelis, 1994; Preston et al., 1995). Our data suggest that HrpZ assists the transfer of AvrB into plant cells and thus may function somewhat like the *Yersinia* YopD protein. YopD is secreted to the bacterial medium, independently possesses cytotoxicity (Hakansson et al., 1993), and is essential for the host cell contact-triggered transfer of other Yop proteins into host cells (Rosqvist et al., 1994; Sory and Cornelis, 1994).

To date, we have been unable to observe secretion of AvrB-FLAG from *P. fluorescens* carrying pHIR11 and pAVRB-FLAG2 or from *P. s. syringae* 61 carrying pAVRB-FLAG2. Immunoblot analyses have shown that bacteria growing in a Hrp-depressing medium (Huynh et al., 1989) secrete HrpZ but not AvrB, and the addition of 0.5% polygalacturonic acid or isolated soybean or tobacco cell walls to the medium failed to trigger release of AvrB (data not shown). This suggests that Avr protein transfer is dependent on a signal generated from contact with living plant cells, and it further implies that the *P. s. syringae* 61 genes directing responsiveness to this signal are carried on pHIR11. Similarly, preliminary experiments with soybean leaves or tobacco suspension cultures, involving lysozyme and proteinase K treatment, have failed to reveal bulk transfer of AvrB-FLAG from bacteria into a protected compartment indicative of localization within plant cells (but transfer of <5% of the AvrB-FLAG probably would have been below detection).

These observations are consistent with the inability of others to observe transfer of Avr proteins from *Xanthomonas* spp into plant cells using immunogold labeling (Brown et al., 1993; Young et al., 1994). Because the volume of a plant cell is four orders of magnitude greater than that of a bacterial cell, the postulated transfer of AvrB into plant cells may be difficult to detect if it involves only a small fraction of the bacterial pool, is transient, or is followed by rapid degradation. Nevertheless, the lack of AvrB action outside of plant cells in the absence of a complete Hrp secretion system, the observed action of AvrB when expressed within plant cells, and the finding that the *RPM1* product (a presumed receptor for AvrB or for an AvrB product generated from plant substrates) is predicted to be in the plant cytoplasm (Grant et al., 1995) provide strong sup-

port for the hypothesis of Hrp-mediated transfer of AvrB. Furthermore, a small fraction of bacterial AvrB transferred into the plant cell may be sufficient to trigger the HR, as suggested by the necrosis in F₁ seedlings of Col × *rps3-1* (*sp-avrB*) line 12, which expressed a very low level of the *avr* transcript (Figure 3E). This model is also consistent with (1) the many observations of highly localized plant cellular responses to pathogens like *P. syringae* (not expected of extracellular elicitors that could diffuse to neighboring plant cells) (Goodman and Novacky, 1994), (2) the recent observations that *P. syringae* Avr signals (including one interacting with RPM1 in Arabidopsis) can interfere with each other outside of the bacterial cell (Reuber and Ausubel, 1996; Ritter and Dangl, 1996), and (3) the determination of genotype-specific incompatibility by single bacterial genes in gene-for-gene interactions (not expected if biosynthetic pathways for low molecular weight elicitors were involved) (Keen, 1990).

P. syringae strains appear to harbor many *avr* genes. This is perhaps most dramatically revealed when genomic libraries of one pathovar are screened in a strain of another pathovar that is normally compatible with a wide range of cultivars of its host (Kobayashi et al., 1989; Dangl, 1994). With the exception of *avrD*, all of the *avr* genes studied are *hrp* dependent in the expression of their genotype-specific phenotypes. Thus, *P. syringae* may transfer many Avr proteins into plant cells via the Hrp secretion pathway. Furthermore, Avr proteins may be only a subset of the transferred bacterial proteins: others that fail to interact with a corresponding resistance gene product would be invisible in current genetic screens. Thus, key challenges for future research are to manipulate the genes and signals controlling the predicted transfer of Avr proteins, thereby unlocking the inventory for inspection in culture, and to explore the biochemical activity of these proteins in plant cells.

It is likely that the original functions of Avr proteins were to promote parasitism; however, defensive selection for less responsive host targets may have diminished their individual contribution to virulence, and the evolution of plant surveillance systems would have rendered these virulence proteins triggers of active plant defense. The proposed function of the Hrp secretion system in delivering bacterial proteins directly into plant cells may have fostered the proliferation of *avr* and resistance genes by permitting economical (and easily replaceable) single gene products to perturb the host metabolism. Thus, acquisition of the type III protein secretion system may have been seminal to the evolution of *P. syringae*-plant interactions to their present level of molecular subtlety and intimacy.

METHODS

Bacterial Strains

Pseudomonas fluorescens and *P. syringae* strains were grown in King's B medium (King et al., 1954) at 30°C or in hypersensitive response (HR) and pathogenicity (Hrp)-derepressing fructose minimal medium

(Huynh et al., 1989). *Escherichia coli* strains were grown routinely in Terrific Broth (Sambrook et al., 1989) or LM medium (Hanahan, 1983) at 37°C, except that MC4100 carrying pHIR11 was grown at 30°C in King's B broth before infiltration into plants. The following bacteria were used in this work: *P. fluorescens* 55, a nalidixic acid-resistant (Nal^r) saprophyte (Huang et al., 1988); *P. syringae* pv *syringae* 61, a wild-type pathogen of bean (Huang et al., 1988); *P. syringae* pv *glycinea* race 4, a wild-type pathogen of soybean (N.T. Keen, University of California at Riverside); *E. coli* DH5 α , *supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nal^r (Hanahan, 1983; Life Technologies, Grand Island, NY); *E. coli* MC4100, F $\bar{\nu}$ Δ (*lac*)*U169 araD136 relA rpsL thi streptomycin resistant* (Oliver and Beckwith, 1981); *E. coli* C2110, PoIA^{ts} (temperature sensitive) Nal^r (Kahn and Hanawalt, 1979); and *P. fluorescens* carrying pCPP2089, which is a *hrcC::TnpA* mutant derivative of pHIR11 (Huang et al., 1991). Antibiotics were used at the following concentrations (μ g/mL): ampicillin (100), kanamycin (50), tetracycline (15), spectinomycin (50), and gentamicin (10).

Construction of pAVRB-FLAG1 and pAVR-FLAG2

Construction of a fusion between the *avrB* avirulence gene and the FLAG synthetic epitope (Hopp et al., 1988) began with polymerase chain reaction (PCR) amplification of *avrB* from pAVRB1 (Tamaki et al., 1988) by using PFU DNA polymerase (Stratagene, La Jolla, CA). The coding strand primer was 5'-CTAGACTCATATGGGCTGCGTCTC-GTC-3' and contained an NdeI site. The reverse strand primer was 5'-CATCATGTGCGACAAAGCAATCAGAATCTAGCAA-3' and contained a Sall site. The amplified DNA was isolated from an agarose gel, digested with NdeI and Sall, ligated into the NdeI-Sall-digested pFLAG-CTC vector (IBI-A Kodak Co., New Haven, CT), and transformed into *E. coli* DH5 α . Transformants were screened for production of the AvrB-FLAG protein by using anti-FLAG M2 antibodies, according to the manufacturer's instructions. pCPP2327 produced AvrB-FLAG and was digested with SspI. The 1.7-kb band containing *avrB*-FLAG was isolated from an agarose gel and ligated into pDSK519 (Keen et al., 1988), which had been digested with XbaI and blunt-ended with the Klenow fragment of DNA polymerase I. *E. coli* DH5 α was transformed, and a plasmid with *avrB*-FLAG in the same orientation as the *lac* promoter was identified by diagnostic restriction digests and designated pAVRB-FLAG1. Production of AvrB-FLAG was confirmed as described above.

To increase the level of AvrB-FLAG expression, pAVRB-FLAG2 was constructed by inserting the SspI fragment from pCPP2327 into pML123 (Labes et al., 1990), which had been digested with BamHI and blunt-ended with the Klenow fragment. AvrB-FLAG2 function was confirmed in *P. s. glycinea* race 4 as described below. In conditions derepressing *hrp* genes, pAVRB-FLAG2 produced \sim 100-fold more AvrB-FLAG than did pAVRB-FLAG1. The relative levels of AvrB-FLAG production by the two plasmids in *P. fluorescens* when grown in Hrp-depressing medium and in soybean leaves were further defined with immunoblots developed for different levels of sensitivity.

Deletion of the *hrpZ* Open Reading Frame from pHIR11

To construct a nonpolar deletion of the entire *hrpZ* open reading frame (ORF), we performed oligonucleotide-directed PCR amplification of flanking sequences followed by direct ligation. A subclone initially containing *hrcJ*-*hrpBZASR* (Alfano et al., 1996) was used (*hrcJ* was previously designated *hrpC* [Bogdanove et al., 1996]). The unmarked mutation was introduced into pHIR11 by successive homologous recom-

bination events occurring at stringent and permissive temperatures fostered by *E. coli* C2110 (PoIA^{ts}), which does not support effective replication of ColE1 replicons at 37°C (Alfano et al., 1996). The resulting mutant, pCPP2274, was confirmed by restriction mapping and PCR analysis. Because *hrpZ* expressed in *trans* interferes with the secretion of any HrpZ made in the cell, we confirmed the nonpolarity of the mutation by its ability to restore HR elicitation activity in tobacco assays to the *hrcJ* mutant *P. s. syringae* 61-79 (Alfano et al., 1996).

Protein Manipulations

AvrB-FLAG was purified to electrophoretic homogeneity by affinity chromatography, using an M2 affinity gel (IBI-A Kodak Co.), as described by the manufacturer. Essentially, 200-mL cultures of *E. coli* DH5 α carrying pAVRB-FLAG2 were grown to an OD₆₀₀ of 0.5 in LM medium (Sambrook et al., 1989) and then induced with isopropyl β -D-thiogalactopyranoside and grown for 5 hr at 37°C. Cells were sonicated in the presence of 1 mM phenylmethylsulfonyl fluoride, and cell-free lysates were loaded onto an M2 affinity column. Following several washes, AvrB-FLAG was eluted with 0.1 M glycine, pH 3.0, and desalted on a Biogel P6DG column (Bio-Rad) equilibrated and eluted with 20 mM Mes, pH 5.6. HrpZ was purified as described previously (He et al., 1993). The AvrB and HrpZ protein preparations each produced a single band on Coomassie Brilliant Blue R 250-stained 12% Phast-Gels (Pharmacia, Uppsala, Sweden). AvrB was infiltrated into plant tissues at a concentration of 0.4 mg/mL. The levels of AvrB produced by pAVRB-FLAG1 and pAVRB-FLAG2 in soybean tissue were determined by excising 1 cm² of infiltrated tissue, lysing all cells in SDS-polyacrylamide gel sample buffer, resolving the released proteins by electrophoresis through a 12% polyacrylamide gel, electrotransferring the proteins to an Immobilon P membrane (Millipore Corp., Bedford, MA), immunoblotting with M2 anti-FLAG antibodies, and then visualizing with the Western Light chemiluminescent assay kit (Tropix Inc., Bedford, MA).

Plant Bioassays

Pseudomonas strains were grown with shaking in King's medium B broth (Huang et al., 1988) to 5×10^8 cells/mL at 30°C. The bacteria were harvested by centrifugation and resuspended in an equal volume of 10 mM MgCl₂. Bacteria were infiltrated into the abaxial surfaces of primary or secondary leaves of soybean (*Glycine max*) by using a No. 5 latex stopper with a hole designed to fit tightly around a 1-mL syringe, thus permitting thumb pressure on the other side of the leaf to provide a tight seal. Soybean plants were grown from seed in pots with Metro 360 Mix (Northeast Horticultural Products, Scotts, Inc., Allentown, PA) in a growth chamber with 14 hr of light at 27°C and 10 hr of darkness at 21°C. Arabidopsis (*Arabidopsis thaliana*) plants were grown with 12 hr of light at 20°C and 70% relative humidity. Leaves of 4- to 5-week-old plants were infiltrated with bacteria by using a syringe without a needle.

Construction of Transgenic *avrB*⁺ Arabidopsis Plants

avrB of *P. s. glycinea* was amplified from pAVRB1 by PCR with an annealing temperature of 52°C. For expression without a signal peptide, primer A (5'-CGGGATCCACCATGGGCTGCGTCTCGTCAAAAAGCACC-3') and primer B (5'-GCTCTAGAT TAAAAGCAATCAGAATCTAGCAAGC-3') were used. For expression with the pathogenesis-related protein

PR-1b signal peptide (Cornelissen et al., 1987), primer B and primer C (5'-TCCGCCGCGGGGGCTGCGTCTCGTCAAAAAGCACC-3') were used. For construction of the PR-1b signal peptide sequence, the following primers were annealed at room temperature: primer D (5'-GAGAAGTGTGCGACACAAGAAAAATGAGGGCATTGTGAAAA-GAG-3'), primer E (5'-GTGTGCGACACTTCTTATTCCTAATAATATCTCACTTCTCTCATGCCCAAAC-3'), primer F (5'-TCCCCGCGGGAG-TTTTGGGCATGAGAAG-3'), primer G (5'-CACGAAGCTTACCATGGA-TTTTTTCTCTTTTAC-3'), and primer H (5'-TCCGCGGGAGTTTG-GGCATGAGAAG-3').

The annealed DNA was purified from an agarose gel using the Prep-A-Gene kit (Bio-Rad), and the PR-1b signal peptide sequence was amplified by PCR using primers G and H (at the annealing temperature of 52°C). The nucleotide sequences surrounding the start codons of *avrB* and the PR-1b signal peptide sequence were modified to A⁻³CCATGG⁺⁴ to conform to the consensus sequence for high-level translation in eukaryotic cells (Kozac, 1984). The *avrB* PCR product was directly cloned into the HindIII site (made blunt following treatment with T4 DNA polymerase) of pKYLX71::35S² (Maiti et al., 1993) for expression without a signal peptide.

For expression with the PR-1b signal peptide, the PCR products of *avrB* and the PR-1b signal peptide sequence were first treated with SacII and XbaI, and HindIII and SacII, respectively, and then ligated into the HindIII-XbaI sites of pKYLX71::35S² as a HindIII-sp-SacII-*avrB*-XbaI fragment. The *avrB* expression plasmids were introduced into *Agrobacterium* strain GV3850 (Zambryski et al., 1983) by triparental mating (Figurski and Helinski, 1979). The plant transformation protocol used was similar to the one described by Bechtold et al. (1993). For gel blot analysis, 10 µg of total RNA was fractionated in agarose-formaldehyde gels and hybridized with a ³²P-labeled *avrB* PCR product at high stringency (Sambrook et al., 1989). Reciprocal crosses were performed between *Arabidopsis rps3-1* (*sp-avrB*) and *Arabidopsis* ecotype Columbia (Col, *GLABROUS1* [*GL1*]) plants. F₁ seeds were plated on plant nutrient agar plates (Bechtold et al., 1993) supplemented with 50 µg/mL kanamycin.

Biolistics and Transient Expression of *avrB* in *Arabidopsis*

Biolistic bombardment of plant leaves was performed using a Bio-Rad PDS-1000/He apparatus and 1300-psi rupture disks. Gold particles (1 µm) were prepared according to the instructions provided by the manufacturer. For each bombardment, 0.5 µg of gold particles was coated with 1 µg of pKYLX71::35S²/*GUS* and 2 µg of pKYLX71::35S² or pKYLX71::35S²/*avrB*. After bombardment, leaves were maintained at 22°C for 24 hr on water-soaked filter paper in Petri dishes. Histochemical β-glucuronidase (*GUS*) staining was done using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate (Seki et al., 1991). The *GUS* gene (in a HindIII-SstI fragment) was cloned into pKYLX71::35S² from pBI101 (Q.-S. Li and S.Y. He, unpublished data).

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