***Pseudomonas* Type III Effector AvrPto Suppresses the Programmed Cell Death Induced by Two Nonhost Pathogens in *Nicotiana benthamiana* and Tomato**

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Many gram-negative bacterial pathogens rely on a type III secretion system to deliver a number of effector proteins into the host cell. Though a number of these effectors have been shown to contribute to bacterial pathogenicity, their functions remain elusive. Here we report that AvrPto, an effector known for its ability to interact with Pto and induce Pto-mediated disease resistance, inhibited the hypersensitive response (HR) induced by nonhost pathogen interactions. *Pseudomonas syringae* pv. *tomato* T1 causes an HR-like cell death on *Nicotiana benthamiana*. This rapid cell death was delayed significantly in plants inoculated with *P. syringae* pv. *tomato* expressing *avrPto*. In addition, *P. syringae* pv. *tabaci* expressing *avrPto* suppressed nonhost HR on tomato *prf3* and *ptoS* lines. Transient expression of *avrPto* in both *N. benthamiana* and tomato *prf3* plants also was able to suppress nonhost HR. Interestingly, AvrPto failed to suppress cell death caused by other elicitors and nonhost pathogens. AvrPto also failed to suppress cell death caused by certain gene-for-gene disease resistance interactions. Experiments with *avrPto* mutants revealed several residues important for the suppression effects. AvrPto mutants G2A, G99V, P146L, and a 12-amino-acid C-terminal deletion mutant partially lost the suppression ability, whereas S94P and I96T enhanced suppression of cell death in *N. benthamiana*. These results, together with other discoveries, demonstrated that suppression of host-programmed cell death may serve as one of the strategies bacterial pathogens use for successful invasion.

Additional keywords: nonhost resistance, VIGS.

Plants have evolved sophisticated defense mechanisms to protect themselves from a vast number of potential pathogens in the surrounding environments. A most emphasized strategy that plants use to deter pathogens is a rapid localized cell death, called the hypersensitive response (HR), which restricts the invading pathogen at the initial infection site. It is widely believed that HR is a type of programmed cell death (PCD) (Heath 2000). The most documented HRs are initiated by the specific recognition of an avirulence gene product (Avr) from the pathogen due to the action of a corresponding plant resistance gene product (R) in a process known as gene-for-gene resistance (Goodman and Novacky 1994). Many patho-

gens also elicit an HR-like host cell death, when inoculated on a nonhost plant, known as nonhost HR (Mysore and Ryu 2004). For example, infiltration of *Pseudomonas syringae* pv. *tomato* into nonhost tobacco leaves leads to a rapid necrosis in the infiltrated area similar to that caused by gene-for-gene induced HR (Keith et al. 2003). Although PCD induced by initiation of gene-for-gene and nonhost interactions may be different, similar mechanisms of PCD led to speculation that there are at least partially conserved pathways involved in both processes (Mysore and Ryu 2004; Thordal-Christensen 2003).

Gram-negative bacterial pathogens have evolved to overcome host defenses using a type III secretion system (TTSS) to deliver a collection of effectors into the host cell milieu (Alfano and Collmer 1997; Chang et al. 2004; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). Translocation of these effectors has been shown to be important for bacterial pathogenicity on hosts (He and Jin 2003). Ironically, most of the effectors initially were identified as avirulence proteins that can elicit HR in host plants containing the corresponding disease resistance (*R*) gene. AvrPto of *P. syringae* pv. *tomato* is a classic example of these effectors. Inoculation of bacterial pathogen *P. syringae* pv. *tomato* expressing *avrPto* onto tomato plants containing the *R* gene *Pto* leads to an HR that results in the restriction of the invading pathogen at the infection site (Martin et al. 1993; Ronald et al. 1992). It has been speculated that AvrPto interacts with Pto, a serine/threonine kinase, inside the host cytosol and leads to the activation of host defense responses (Scofield et al. 1996; Tang et al. 1996, 1999). Activation of Pto-AvrPto mediated defense responses also requires a second host gene, *Prf* (Salmeron et al. 1996). Further genetic analysis of AvrPto indicated that its function extends beyond its ability to induce host defense. AvrPto enables bacterial pathogens to achieve optimal growth on susceptible tomato plants, thus providing a fitness advantage for the bacteria (Chang et al. 2000; Shan et al. 2000b). Expression of *avrPto* in the plant cell particularly suppressed a set of cell wall-based defense genes (Hauck et al. 2003). Though AvrPto's function in promoting disease in susceptible hosts has been reported, the mechanisms by which it acts remain largely unknown.

Recently, a series of reports demonstrated that several effector proteins were able to suppress host PCD during a disease resistance response. These findings provide a plausible explanation for the function of effectors in bacterial pathogens. For example, AvrPtoB, an effector protein from *P. syringae* pv. *tomato*, was shown to inhibit PCD in tobacco and yeast (Abramovitch et al. 2003). Interestingly, AvrPtoB shares

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52% amino acid similarity with the effector VirPphA from *P. syringae* pv. *phaseolicola*, which also enables this bacterial strain to evade an HR-based resistance in bean (Jackson et al. 1999). In addition, Espinosa and coworkers showed that another effector, HopPtoD2, from *P. syringae* pv. *tomato* strain DC3000, suppressed HR and oxidative burst in *Nicotiana benthamiana* (Espinosa et al. 2003). Furthermore, five effector proteins from *P. syringae* pv. *tomato* DC3000 also were reported to be able to suppress PCD in a trans-kingdom manner (Jamir et al. 2004). These discoveries highlight the evolutionary significance of type III effectors in bacterial pathogenicity, and imply that the suppression of PCD is a possible strategy employed by the bacterial pathogen to ensure its survival in the host plant.

Here we report that AvrPto, an effector from *P. syringae* pv. *tomato*, acts as an inhibitor of host PCD caused by specific pathogen and nonhost plant interactions. By using nonhost HR as an indicator, we also identified regions of AvrPto that are important for this inhibition effect. These results provide additional evidence for a virulence activity of AvrPto. The approaches reported in this article also could be expanded to studies of other effectors and help us to gain insight on the evolution and importance of bacterial effector proteins and nonhost resistance.

RESULTS

AvrPto delays nonhost HR triggered

by *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci*.

During the studies of AvrPto and Pto-mediated resistance responses, we observed that the expression of *avrPto* consistently inhibited nonhost HR triggered by two *Pseudomonas* strains. *P. syringae* pv. *tomato* strain T1 produced a nonspecific HR within 20 h when infiltrated into leaves of the non-host plant *N. benthamiana* at a concentration of 2×10^7 CFU/ml (Fig. 1A). Interestingly, infiltration with *P. syringae* pv. *tomato* strain T1A, which expresses *avrPto* (Ronald et al. 1992), into *N. benthamiana* did not induce an HR until at least 36 h after inoculation. This delay in the cell death lasted from 16 to 48 h and varied by leaf age and surrounding temperature. Whole *N. benthamiana* plants, when vacuum infiltrated with *P. syringae* pv. *tomato* strain T1 or T1A at 2×10^7 CFU/ml, developed HR on whole plants within 24 h after inoculation with T1 but the HR was delayed until 48 h after inoculation with T1A (Fig. 1B). Interestingly, the HR development was much faster on the older, bottom leaves than the younger, top leaves.

We expanded our study to the interaction of tomato and *P. syringae* pv. *tabaci* to further determine the effect of AvrPto on suppression of nonhost HR. Tomato line Rio Grande-prf3 is

Fig. 1. Suppression of nonhost hypersensitive response (HR) by AvrPto in *Nicotiana benthamiana* and tomato. **A**, *Pseudomonas syringae* pv. *tomato* strains T1 and T1A (expressing *avrPto*) were injected into symmetrical sides of *N. benthamiana* leaf at a concentration of 2×10^7 CFU/ml. HR developed on the T1-inoculated side but not on T1A-inoculated side. Picture was taken 24 h after inoculation. **B**, Whole *N. benthamiana* plants were inoculated (vacuum infiltration) with T1A and T1 (2×10^7 CFU/ml). T1 caused more cell death on old (bottom) leaves (see insert). Photograph was taken 24 h after inoculation. **C**, *P. syringae* pv. *tabaci* and *P. syringae* pv. *tabaci*(pPTE6::avrPto) were injected into symmetrical sides of a prf3 tomato leaf at a concentration of 2×10^7 CFU/ml. HR developed on the *P. syringae* pv. *tabaci*-inoculated side but not on the *P. syringae* pv. *tabaci*(pPTE6::avrPto) side 24 h after inoculation. **D**, Transient expression of *avrPto* was sufficient to suppress nonhost HR. *Agrobacterium tumefaciens* GV2260 expressing either the empty vector or *avrPto* (optical density at 600 nm = 0.4) was infiltrated into *N. benthamiana* leaf areas marked as the red outer circle 24 h prior to *Pseudomonas* inoculation. The inner black circles are regions infiltrated with either *P. syringae* pv. *tomato* (T1) or *P. syringae* pv. *tabaci* at 2×10^7 CFU/ml. Top panels indicate vector control. Lower panels were taken from *avrPto*-expressing leaves. Photograph was taken 24 h after pathogen inoculation.

defective in the *Pto*-mediated signaling pathway because of a deletion in *Prf* (an essential component of *Pto*-mediated resistance) (Salmeron et al. 1996) and is a nonhost for the tobacco pathogen *P. syringae* pv. *tabaci*. When inoculated with *P. syringae* pv. *tabaci* at a concentration of 2×10^7 CFU/ml, nonhost HR developed on prf3 tomato leaves within 24 h after inoculation (Fig. 1C). We transferred pPTE6::*avrPto* (the broad host-range pDSK519 plasmid containing *avrPto*) (Ronald et al. 1992) into *P. syringae* pv. *tabaci*. When inoculated with *P. syringae* pv. *tabaci* expressing *avrPto*, prf3 tomato plants produced delayed (by 6 to 16 h) nonhost HR compared with *P. syringae* pv. *tabaci* without *avrPto*. To check whether AvrPto can be delivered from *P. syringae* pv. *tabaci* into the plant cell, we inoculated tobacco W38 (contains an active *Pto* homolog) (Thilmony et al. 1995) plants with *P. syringae* pv. *tabaci* expressing AvrPto. We observed a rapid HR within 18 h after infiltration due to AvrPto-*Pto* recognition, whereas *P. syringae* pv. *tabaci* without AvrPto caused disease 36 h after infection (data not shown). The HR inhibition effect also was observed on RioGrande-*ptoS* tomato lines (does not have an active *Pto* gene) but the suppression lasted longer on prf3 lines compared with *ptoS* lines. Inhibition of cell death also was observed on nonhost pepper (*Capsicum annuum* cv. Marengo) and *Arabidopsis* (Col-0) plants inoculated with *P. syringae* pv. *tomato* or *P. syringae* pv. *tabaci* strains expressing AvrPto. However, the suppression effect was not as evident as on *N. benthamiana* and tomato plants (data not shown). These data suggest that AvrPto can suppress cell death related to nonhost HR.

Transient expression of AvrPto results in delay of nonhost HR.

To further test whether AvrPto alone is the factor that contributed to the cell death inhibition observed during nonhost interactions, we conducted *Agrobacterium*-mediated transient expression assays to express AvrPto in planta as reported previously (Sessa and Martin 2000). Several dilutions of *Agrobacterium* spp. containing the pBtex binary vector or pBtex::*avrPto* (Frederick et al. 1998) were infiltrated into leaves of *N. benthamiana* and prf3 tomato plants. At 24 h after agro-infiltration, suspensions of *P. syringae* pv. *tomato* strain T1 and *P. syringae* pv. *tabaci* were injected onto a smaller area within the sites previously inoculated with *Agrobacterium* spp. on *N. benthamiana* plants and tomato prf3 plants, respectively. A typical HR developed 24 h after inoculation with *P. syringae* pv. *tomato* strain T1 on the leaves of *N. benthamiana* pre-inoculated with *Agrobacterium* spp. containing the empty vector (Fig. 1D). However, those leaves transiently expressing *avrPto* remained green, and no visible HR was observed until 48 h after inoculation. The effect of cell death suppression positively correlated with the concentration of *Agrobacterium* spp. used in the experiments, which presumably correlates with the amount of AvrPto produced in the plant cell (data not shown). The strongest suppression effect was observed on leaves inoculated with *Agrobacterium* spp. concentration at 4×10^8 CFU/ml, which sustained at least an additional 24 h. Similar results were observed with *P. syringae* pv. *tabaci* on prf3 tomato plants, although the suppression did not last longer than 36 h after pathogen inoculation in all replicates (Fig. 1D). Surprisingly, in both the experiments with *N. benthamiana* and prf3 tomato, the suppression effect did not sustain as long as those caused by *Pseudomonas* bacteria expressing *avrPto*. This probably is because additional factors from the bacteria involved in this process are missing or the AvrPto protein expressed inside the host cell might not be as stable as those delivered by the bacterial TTSS. Nevertheless, this experiment demonstrated that AvrPto alone can inhibit nonhost HR triggered by the two pathogens examined.

Suppression of cell death is not due to Pto-AvrPto-mediated resistance.

P. syringae pv. *tomato* strain T1A and *P. syringae* pv. *tabaci*(pPTE6::*avrPto*) were able to elicit a resistance response (HR), due to *Pto*-AvrPto interaction, in tomato *PtoR* and *N. tabacum* W38 plants, respectively, within 18 h after inoculation, as previously reported (data not shown) (Shan et al. 2000b). It is possible that the cell death we observed during nonhost and *Pseudomonas* spp. interactions could be due to disease and the suppression effects could be from activation of weak *Pto*-mediated resistance. *Pto* homologs are known to be present in several Solanaceous species, including *N. benthamiana* (Grube et al. 2000; G. Martin, personal communication). To rule out this possibility, we compared growth of both *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci* with or without *avrPto* in corresponding host and nonhost plants. Three days after pathogen challenge, *P. syringae* pv. *tomato* strains T1 and T1A did not show significant difference in growth, although the population of both bacteria was increased slightly in nonhost *N. benthamiana* plants 3 days after inoculation (Fig. 2A). Consistent with previous reports (Chang et al. 2000; Shan et al. 2000a), T1 and T1A populations grew to at least 2×10^7 CFU/cm² in susceptible prf3 tomato plants, while the T1A population grew approximately fivefold higher (Fig. 2B). Similarly, a population of *P. syringae* pv. *tabaci* with or without *avrPto* grew to at least 2×10^7 CFU/cm² in susceptible *N. benthamiana* leaves (Fig. 2C). Both bacterial populations grew slightly in nonhost prf3 tomato plants 3 days after inoculation. Interestingly, *P. syringae* pv. *tabaci* with *avrPto* grew consistently 5- to 10-fold higher in tomato prf3 plants than the wild-type *P. syringae* pv. *tabaci* (Fig. 2D). This is consistent with a previous report indicating that *avrPto* could be a virulence factor (Chang et al. 2000). Nevertheless, the presence of *avrPto* in *Pseudomonas* spp. did not result in a lower bacterial population or slower growth; thus, we can rule out the possibility of *Pto*-AvrPto-mediated resistance playing a role in suppression of cell death.

To further confirm that the *Pto*-AvrPto-mediated resistance does not play a role in suppression of cell death, we silenced the *N. benthamiana* homolog of the tomato *Prf* gene using virus-induced gene silencing (VIGS). *Prf*, a nucleotide binding site leucine-rich repeater protein is required for the activation of *Pto*-mediated disease resistance. Silencing of *Prf* by VIGS in *N. benthamiana* has been shown to abolish *Pto*-AvrPto-mediated HR (Jin et al. 2002; Lu et al. 2003; Peart et al. 2002a). We used *Tobacco rattle virus* (TRV)-derived vectors for VIGS (Liu et al. 2000a,b). Two weeks after inoculation with TRV-RNA1 and TRV-RNA2::*Prf*, systemic silencing of *Prf* occurred on the upper part of *N. benthamiana* plants. Although the HR induced due to *Agrobacterium*-mediated transient expression of *avrPto* and *Pto* was interrupted in the silenced plants, suppression of nonhost HR remained after T1A inoculation (Fig. 3A).

Suppression of cell death by AvrPto is limited to only the two tested nonhost interactions.

The *Pseudomonas* type III effector AvrPtoB has been shown to generally suppress PCD in *N. benthamiana* (Abramovitch et al. 2003). To test whether AvrPto also can suppress general PCD, we expanded our study with several other nonhost bacterial strains. *Agrobacterium*-mediated transient assay was used to express *avrPto* in planta in *N. benthamiana*. At 24 h after inoculation with *Agrobacterium* spp., nonhost bacteria *Xanthomonas axonopodis* pv. *vesicatoria*, *Erwinia amylovora* Ea73, *P. syringae* pv. *syringae*, and *P. syringae* pv. *lachrymans* were infiltrated at a concentration of 2×10^7 CFU/ml into the same area inoculated with *Agrobacterium* spp. Transient expression of *avrPto* did not delay HR induced by *E. amylovora*, *X. axono-*

podis pv. *vesicatoria*, or *P. syringae* pv. *syringae*, whereas severity of HR caused by *P. syringae* pv. *lachrymans* was slightly reduced compared with control plants inoculated with *Agrobacterium* spp. containing empty vector (Fig. 3B).

We also examined whether AvrPto would affect several other types of cell death resulting from different gene-for-gene interactions and cell death-inducing chemicals. Coexpression of *Avr9* and *Cf9* results in a gene-for-gene-induced HR in *N. benthamiana* (Abramovitch et al. 2003; Peart et al. 2002b; Thomas et al. 2000). Expression of fungal elicitor *inf1*, the bacterial effector *avrRpt2*, or a *Pto* mutant (Y207D) in *N. benthamiana* previously was shown to cause HR-like cell death (Kamoun et al. 1998; Mudgett and Staskawicz 1999; Rathjen et al. 1999). Chemicals such as 500 mM NaCl, 20% ethanol, and 3% H₂O₂ also have been shown to cause cell death in *N. benthamiana* (Peart et al. 2002b). AvrPto did not suppress these cell death processes (Fig. 3C and D). These results indicate that the suppression of PCD by AvrPto is specific to certain nonhost interactions.

Mutants of AvrPto alter cell death suppression effects.

To test whether there are important regions or domains in AvrPto protein that are critical for its HR suppression effects in nonhost plants, we tested six AvrPto mutants that have been shown to affect virulence and avirulence functions (Shan et al. 2000a and b; Tang et al. 1996) (Table 1). Surprisingly, all six

AvrPto mutants tested exhibited different suppression effects than the wild-type AvrPto. Four AvrPto mutants (G2A, G99V, P146L, and a C-terminal 12 amino acid deletion mutant [CD12]) showed partially disrupted cell death inhibition effects on *N. benthamiana* after nonhost pathogen inoculation although they all still caused a reduced HR compared with those caused by T1. All T1-inoculated leaf area showed severe cell death, whereas leaves inoculated with T1A remained healthy 24 h after leaf infiltration (Fig. 4). The G99V mutation had the most reduced effect on suppression among the tested mutants; more than two-thirds of inoculation sites showed mild cell death, though the severity of HR was less compared with T1. G2A, P146L, and CD12 mutants caused intermediate HR on approximately 50% of inoculated leaves. All symptoms were greatly enhanced 48 h after inoculation. Interestingly, two other AvrPto mutants (S94P and I96T) showed enhanced suppression effects. At 48 h after inoculation, cell deaths were observed on approximately half of leaves injected with T1A. Almost all the leaves expressing the *avrPto* mutants S94P and I96T remained healthy and only 1 of 15 injected leaves showed slight cell death. Together, these results showed that there are residues or regions in AvrPto that are critical for its normal function of suppressing nonhost cell death. Presumably, disruption of these residues or regions also may affect the ability of AvrPto to interact with targets in host plants involved in regulating cell death processes.

Fig. 2. In planta growth of *Pseudomonas*-expressing *avrPto* was comparable to or better than that of those that do not express *avrPto*. Bars marked with asterisk denotes significant difference at $P = 0.05$. **A**, Growth of *Pseudomonas syringae* pv. *tomato* strains T1 and T1A (expresses *avrPto*) in *Nicotiana benthamiana*; **B**, growth of T1 and T1A in *prf3* tomato lines; **C**, growth of *P. syringae* pv. *tabaci* and *P. syringae* pv. *tabaci*(pPTE6::*avrPto*) in *N. benthamiana*; **D**, growth of *P. syringae* pv. *tabaci* and *P. syringae* pv. *tabaci*(pPTE6::*avrPto*) in *prf3* tomato plants.

Fig. 3. Suppression of cell death by AvrPto is not dependent on *Pto*-mediated resistance pathway and is specific only to certain nonhost interactions. **A**, The *Prf* gene (required for *Pto*-mediated resistance) was silenced using a *Tobacco rattle virus* (TRV)-based virus-induced gene silencing system. *Agrobacterium* strain GV2260 containing TRV2-*Prf* or empty vector (TRV2::00) were infiltrated, along with *Agrobacterium* containing TRV1, into leaves of 3-week-old *Nicotiana benthamiana*. Three weeks after TRV inoculation, *Agrobacterium* strains containing pBtex::Pto and pBtex::avrPto were mixed at a 1:1 ratio to a final concentration of 4×10^8 CFU/ml for each construct and infiltrated. The hypersensitive response caused by Pto and AvrPto was photographed 4 days after inoculation. *Pseudomonas syringae* pv. *tomato* strains T1 and T1A (expresses *avrPto*) were inoculated at 2×10^7 CFU/ml. Pictures were taken 24 h after inoculation. **B**, Nonhost pathogens *Xanthomonas axonopodis* pv. *vesicatoria*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *lachrymans*, and *Erwinia amylovora* (Ea73) were infiltrated at 2×10^7 CFU/ml into the smaller circled areas of leaves 24 h after inoculation with *Agrobacterium* spp. (outer circle) containing *avrPto* (top panel) or vector control (bottom panel). Photograph was taken 24 h after pathogen infiltrations. **C**, *avr9-cf9*, *inf1*, *avrRpt2*, *Pto*^{Y207D}, and *Pto* were coexpressed with *avrPto* (top panel) or vector control (bottom panel) by *Agrobacterium* spp. in *N. benthamiana* as described in B. Photograph was taken 4 days after *Agrobacterium* infiltration. **D**, Cell death-inducing chemicals H₂O₂ (3%), ethanol (20%), and NaCl (500 mM) were infiltrated into the smaller circled areas of leaves 24 h after inoculation with *Agrobacterium* spp. containing *avrPto* (top panel) or vector control (bottom panel). Photograph was taken 24 h after chemical infiltrations.

DISCUSSION

Our understanding of plant disease resistance and pathogenicity has been greatly enhanced during the past decade. TTSS effectors have been shown to be crucial pathogenicity factors in many plant-pathogenic bacteria (Galan and Collmer 1999; Petnicki-Ocwieja et al. 2002). Although a large number of TTSS effectors have been identified, it remains an intriguing challenge to unravel their functions in causing disease. AvrPto from *P. syringae* pv. *tomato* is a classical example of such effector proteins. In this work, we showed that AvrPto suppressed nonhost pathogen-induced cell death in *N. benthamiana* and tomato *prf3* plants caused by *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci*, respectively. *Agrobacterium*-mediated transient expression of *avrPto* was sufficient to suppress

cell death caused by a nonhost pathogen. Although AvrPto’s ability to elicit host resistance responses is well studied, this report on AvrPto suppressing cell death adds to its enigmatic functions in bacterial pathogenesis.

We showed that the suppression of cell death process was not likely due to activation of *Pto*-mediated resistance, through AvrPto–Pto (homolog) interaction, in both tomato and *N. benthamiana*. In the absence of *Prf*, an essential component of *Pto*-mediated resistance (Salmeron et al. 1996), the *Pto*-mediated resistance pathway was not activated, but the suppression of cell death by AvrPto remained. We also showed that *Pseudomonas* spp. expressing *avrPto* grew to a similar or slightly higher population compared with *Pseudomonas* strains without *avrPto* in nonhost plants. It is interesting to note that *P. syringae* pv. *tabaci* expressing *avrPto* consistently grew 5- to 10-

Table 1. Summary of AvrPto mutants tested in this study^a

AvrPto mutant	Pto interaction	Secretion in MM	Protein stability	Putative function, virulence, and avirulence activities	Reference
G2A	Yes	Yes	Stable	Mutation of the putative myristoylation site for membrane anchor; disrupts avirulence and virulence activities	Shan et al. 2000b
S94P, I96P, G99V	No	Yes	Stable	Specifically disrupt the Pto-interaction and avirulence in tomato; have virulence activity in tomato and avirulence in tobacco	Shan et al. 2000b
P146L	Yes	Yes	Stable	Specifically disrupts avirulence in tobacco; reduces avirulence in Pto-tomato; slightly reduces virulence in tomato	Unpublished
CD12	Yes	ND	ND	Specifically disrupts avirulence in tobacco; reduces avirulence in Pto-tomato; virulence not determined	Shan et al. 2000b; Tang et al. 1996

^a MM = minimal medium; ND = not determined.

Fig. 4. Regions and residues of AvrPto that are important for cell death suppression. *Nicotiana benthamiana* leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* strains, expressing various *avrPto* mutants (shown on top), at 2×10^7 CFU/ml. *P. syringae* pv. *tomato* strains T1 and T1A (expressing wild-type *avrPto*) were used as controls. Photographs were taken 24 h after inoculation and only representing phenotypes are shown. Single-letter symbols are used to name amino acids. The CD12 AvrPto mutant has a C-terminal deletion of 12 amino acids. The numbers on the bottom of the photographs denote the number of leaves that showed cell death versus the number of leaves inoculated at 24 and 48 h after inoculation.

fold higher in nonhost tomato plants compared with *P. syringae* pv. *tabaci* without *avrPto*. This is similar to the phenomenon observed in the susceptible tomato plants, where *avrPto*-expressing *Pseudomonas* spp. enable a similar magnitude increase of bacterial population (Chang et al. 2000; Shan et al. 2000a). Among the several *AvrPto* mutants we tested, S94P and I96T mutants, that do not interact with Pto in vitro (Shan et al. 2000b), maintained cell death inhibition effects on *N. benthamiana*. These evidences again emphasize the virulence activity of *AvrPto* in the absence of its corresponding Pto-mediated resistance, although the mechanisms of this virulence activity still remain unknown.

It is tempting to speculate that *AvrPto* interacts with one or more host factors that regulate host cell death processes to inhibit nonhost HR. To address this possibility, we asked whether different domains of *AvrPto* are important for its inhibition effects. We hypothesize that mutations in different domains of *AvrPto* would affect its ability to interact with its presumable host target or targets involved in cell death regulation. Several *AvrPto* mutants that do not interact with Pto have been identified previously (Shan et al. 2000b). Interestingly, even though these mutants lost avirulence function, many of them maintained the virulence function (Shan et al. 2000a and b). Among the six mutants we tested (Table 1), five *AvrPto* mutants partially lost cell death inhibition effects. P146L and CD12 mutants have mutations in the C-terminal region of *AvrPto* protein that is required for avirulence activity in tobacco (Shan et al. 2000a). The cell death suppression was partially impaired by these two mutants and exhibited different degrees of suppression on *N. benthamiana* plants (Fig. 4). G2A mutation has been shown to abolish *AvrPto* membrane association (Shan et al. 2000b) and the suppression activity of this mutant was partially disrupted. This led us to postulate that suppression of cell death might require correct subcellular localization of *AvrPto* in the host cell. To our surprise, two mutants (S94P and I96T) showed enhanced inhibition effects on *N. benthamiana*. Interestingly, the *P. syringae* pv. *tomato* strains expressing these two mutant *avrPto* also were shown to exhibit higher bacterial numbers than T1 in both ptoS and PtoR plants (Shan et al. 2000a). Surprisingly, the G99V mutant showed the least inhibition effect and the mutation was only a few amino acids away from S94P and I96T mutations. Furthermore, all three mutants showed similarly disrupted avirulence activity in tomato plants but not in tobacco (Shan et al. 2000b). These three mutations did not affect secretion of *AvrPto* from bacteria in vivo (Shan et al. 2000b). It is possible that this central region of *AvrPto* (amino acids 94 to 99) plays a critical role in suppression of cell death, although further analysis of more mutants is required to test this hypothesis. These results further demonstrate that the avirulence and virulence functions of *AvrPto* can be structurally separated.

Much evidence from our research implied that there are host factors involved in the suppression of cell death by *AvrPto*. The suppression effect lasted significantly longer in younger and new-emerging leaves than in older leaves. The suppression also was affected by temperature and other growth conditions, suggesting that potential host targets also may be involved in plant development. When suppression effects were compared between tomato and *N. benthamiana*, the suppression effects were much stronger in *N. benthamiana*; this probably is due to the differences in host factors in these two plants. It is important to note that, using a yeast two-hybrid approach, *AvrPto*-interacting tomato proteins have been identified earlier (Bogdanove and Martin 2000). Most likely *AvrPto*, in the absence of Pto, interacts with host proteins and contributes to pathogenicity. In an effort to iso-

late host factors involved in *AvrPto*-mediated cell death inhibition, we currently are performing fast-forward genetics using VIGS in *N. benthamiana*.

Suppression of host PCD recently emerged as one of the strategies for bacterial invasion in plants. As biotrophic pathogens, *Pseudomonas* bacterial pathogens need to acquire nutrients from living hosts. It is speculated that HR or PCD in the host limits the resources available for bacterial survival. By blocking the HR processes, bacteria will be able to secure nutrition, resulting in their successful multiplication. There also is evidence suggesting that the reduction of water potential during HR could limit *P. syringae* pv. *tomato* growth (Wright and Beattie 2004). The HR often is used as a defense to restrict invading bacterial pathogens; therefore, it is plausible that TTSS effectors would target this process. It has been shown that a *Pseudomonas* TTSS effector *AvrPtoB* suppresses the *Avr-R* protein-induced HR, Bax-induced cell death in plants, and heat-induced PCD in yeast (Abramovitch et al. 2003). *Pseudomonas* TTSS effector proteins *AvrPtoB*, *AvrPphE_{Pto}*, *AvrPpiB1_{Pto}*, *HopPtoG*, *HopPtoF*, and *HopPtoE* also have been shown to suppress *HopPsyA*-dependent HR in tobacco and *Arabidopsis* spp. (Jamir et al. 2004). Interestingly, *AvrPtoB* and *AvrPpiB1_{Pto}* failed to suppress Bax-induced yeast PCD, whereas the others did. Surprisingly, in this work, we show that *AvrPto* only suppressed cell death caused by certain non-host interactions. Due to the complexity of cell death regulation in the host plant, it is not surprising that various effectors would have different targets and, therefore, exhibit various specificities. These observations suggest that suppressing host PCD seems to be one of the strategies adapted by bacterial pathogens to ensure a successful infection.

However, the suppression of nonhost HR by *AvrPto* doesn't last for more than 3 days. We hypothesize that more than one bacterial effector and interaction of bacterial effectors with certain plant proteins is required for stable suppression of non-host HR. For example, *AvrPto* from *P. syringae* pv. *tomato* can initiate cell death suppression in the nonhost plant *N. benthamiana*. However, the suppression effect doesn't last longer because the right combination of additional effectors from *P. syringae* pv. *tomato* and plant factors that are required for suppression of nonhost HR in *N. benthamiana* are missing. Similarly, the suppression of cell death due to *P. syringae* pv. *tabaci* infection on nonhost tomato plants also can be initiated by *AvrPto*, but the right combination of additional effectors from *P. syringae* pv. *tabaci* and plant factors that are required for stable suppression of nonhost HR in tomato are missing. It is interesting to note that *P. syringae* pv. *tabaci* expressing *avrPto* can grow approximately 5- to 10-fold higher in nonhost tomato plants compared with wild-type *P. syringae* pv. *tabaci* (Fig. 2D). Transient suppression of nonhost HR by *AvrPto* could be contributing to this increase in bacterial population.

It is a known fact that the plants normally are resistant to most potential pathogens. One of the mechanisms by which plants resist many potential pathogens is type II nonhost resistance that causes HR upon infection (Mysore and Ryu 2004). Some bacterial pathogens have evolved to suppress this non-host HR by targeting the host cellular machinery through its effector proteins. We speculate that a successful bacterial pathogen that can cause plant disease may have effectors that can interact with plant proteins to suppress HR caused by host and nonhost resistances.

MATERIALS AND METHODS

Bacterial strains, media, and growth.

Agrobacterium tumefaciens strain GV2260 was grown in LB medium containing the proper antibiotics at 30°C. *P. syrin-*

gae pv. *tomato* strains T1 and T1A (pPTE6::avrPto[mutants]) and *P. syringae* pv. *tabaci* were grown in King's B (KB) medium containing the proper antibiotics at 30°C. Broad-host-range plasmid pPTE6::avrPto was introduced into *P. syringae* pv. *tabaci* by electroporation. Correlation of bacterial suspension turbidity at an optical density at 600 nm (OD₆₀₀) and CFU per milliliter was established by serial plating. Under the experimental conditions, *P. syringae* pv. *tabaci* at 1_{OD600} equals 2 × 10⁸ CFU/ml and *P. syringae* pv. *tomato* at 1_{OD600} equals to 1 × 10⁸ CFU/ml. Correlation of values at OD₆₀₀ and bacterial concentrations of the wild type-strain and *avrPto*-plasmid-containing strains did not show significant differences. Antibiotics were used at concentrations of rifampicin at 100 µg/ml and kanamycin at 50 µg/ml.

In vivo bacterial growth assay.

Whole plants were vacuum infiltrated with 2 × 10⁴ CFU/ml bacterial suspensions in 10 mM MgCl₂. To facilitate infiltration, Silwet L-77 (Osi Specialties, Friendship, WV, U.S.A.) was added to the bacterial suspension to a final concentration of 0.01%. Three days after inoculation, leaf discs were harvested, ground in 10 mM MgCl₂, and CFU per square centimeter was determined by plating serial dilutions of leaf extracts on *Pseudomonas* agar F (Fisher Co., Hampton, NH, U.S.A.) plates containing appropriate antibiotics. Each data point represents average bacterial numbers of at least four replicates and error bars indicate standard deviations. Data were subjected to analysis of variance using JMP software (version 4.0.4; SAS Institute Inc., Cary, NC, U.S.A.). When a significant *t* test was obtained at *P* = 0.05, separation of treatment means was accomplished by Fisher's protected least significant difference (LSD). The experiments were repeated at least three times with similar results and representative results are shown.

VIGS assay for silencing *Prf* in *N. benthamiana*.

The *A. tumefaciens* strains GV2260 containing TRV-VIGS vectors were used for VIGS experiments (Liu et al. 2000a,b). An *Agrobacterium* sp. containing a TRV2-*Prf* clone was obtained from G. Martin, BTI. Bacterial cells were harvested by centrifugation of overnight liquid culture at 4,000 rpm, washed once in 10 mM MES (morpholineethanesulfonic acid) (pH 5.6), resuspended in *Agrobacterium* inoculation buffer (10 mM MgCl₂, 10 mM MES [pH 5.6], 200 µM acetosyringone) to a final concentration of 1 × 10⁸ CFU/ml (for both TRV1 and TRV2), and shaken for 4 h at room temperature before infiltration. *Agrobacterium* strains containing TRV1 and TRV2::prf or vector alone were mixed in a 1:1 ratio and infiltrated into the leaves of 2- to 3-week-old plants with a 1-ml needleless syringe. Two weeks after inoculation, silencing of *Prf* in *N. benthamiana* was confirmed by HR assay with transient coexpression of *avrPto* and *Pto* (Sessa et al. 2000).

Plant inoculation and nonhost HR assay.

For the nonhost HR assay, bacterial cells were collected by centrifuging overnight liquid culture at 4,000 rpm, washed three times with 10 mM MgCl₂, and resuspended in 10 mM MgCl₂ to appropriate concentrations. Then, 4- to 5-week-old *N. benthamiana* plants and 3- to 4-week-old tomato RioGrande-prf3, -ptoS, and -PtoR plants, grown in the greenhouse, were infiltrated on young leaves with appropriate bacterial suspensions using a needleless syringe. For nonhost HR assays on pepper and *Arabidopsis*, bacterial concentrations of 2 × 10⁷ CFU/ml were used. To eliminate developmental differences, a wild-type strain and strains containing *avrPto* were inoculated on symmetrical sides of the same leaf. After inoculation, plants were kept at room temperature for symptom development.

Agrobacterium-mediated transient assays.

Transient expression of *avrPto* and *Pto*, both driven by CaMV 35S promoter, were performed as previously described (Sessa et al. 2000). In brief, overnight cultures of *A. tumefaciens* GV2260 containing pBtex::avrPto, pBtex::Pto, and vector alone were washed once in 5 mM MES (pH 5.6) and resuspended in 5 mM MES to desired concentrations. Acetosyringone was added to the bacterial suspension to the final concentration of 200 µM before infiltrating into leaves of *N. benthamiana* or prf3 tomato plants with a needleless syringe. At 24 h after *Agrobacterium* inoculation, wild-type *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci* were infiltrated into a smaller area within the same inoculation site previously inoculated with *Agrobacterium* spp. In two independent experiments with at least four replicates in each, similar results were observed and only representing data are shown.

For Cf-9+Avr9, Inf1, Pto, Pto (Y207D), and AvrRpt2 HR assays, all genes (driven by CaMV 35S promoter) were expressed transiently by *Agrobacterium* spp. as reported previously (Abramovitch et al., 2003; Peart et al. 2002b). After *Agrobacterium* suspensions were prepared as described above, bacteria were mixed with *Agrobacterium* GV2260 containing pBtex::avrPto or empty vector to a final concentration of 2 × 10⁸ CFU/ml and coinfiltrated into leaves of *N. benthamiana*.

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LITERATURE CITED

- Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B., and Martin, G. B. 2003. *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO (Eur. Mol. Biol. Organ.) J. 22:60-69.
- Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. J. Bacteriol. 179:5655-5662.
- Bogdanove, A. J., and Martin, G. B. 2000. AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. Proc. Natl. Acad. Sci. U.S.A. 97:8836-8840.
- Chang, J. H., Goel, A. K., Grant, S. R., and Dangel, J. L. 2004. Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria. Curr. Opin. Microbiol. 7:11-18.
- Chang, J. H., Rathjen, J. P., Bernal, A. J., Staskiewicz, B. J., and Michelmore, R. W. 2000. AvrPto enhances growth and necrosis caused by *Pseudomonas syringae* pv. *tomato* in tomato lines lacking either *Pto* or *Prf*. Mol. Plant-Microbe Interact. 13:568-571.
- Espinosa, A., Guo, M., Tam, V. C., Fu, Z. Q., and Alfano, J. R. 2003. The *Pseudomonas syringae* type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. Mol. Microbiol. 49:377-397.
- Frederick, R. D., Thilmony, R. L., Sessa, G., and Martin, G. B. 1998. Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase. Mol. Cell 2:241-245.
- Galán, J. R., and Collmer, A. 1999. Type III secretion machines: bacterial device for protein delivery into host cells. Science 284:1322-1328.
- Goodman, R. N., and Novacky, A. J. 1994. Pages 7-8 in: The Hypersensitive Reaction in Plants to Pathogens. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Grube, R. C., Radwanski, E. R., and Jahn, M. 2000. Comparative genetics of disease resistance within the *Solanaceae*. Genetics 155:873-887.
- Guttman, D. S., Vinatzer, B. A., Sarkar, S. F., Ranall, M. V., Kettler, G., and Greenberg, J. T. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. Science 295:1722-1726.
- Hauck, P., Thilmony, R., and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in sus-

- ceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:8577-8582.
- He, S. Y., and Jin, Q. 2003. The Hrp pilus: learning from flagella. *Curr. Opin. Microbiol.* 6:15-19.
- Heath, M. C. 2000. Hypersensitive response-related death. *Plant Mol. Biol.* 44:321-334.
- Kamoun, S., van West, P., Vleeshouwers, V. G. A. A., de Groot, K. E., Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* 10:1413-1425.
- Jackson, R. W., Athanassopoulos, E., Tsiamis, G., Mansfield, J. W., Sesma, A., Arnold, D. L., Gibbon, M. J., Murillo, J., Taylor, J. D., and Vivian, A. 1999. Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*. *Proc. Natl. Acad. Sci. U.S.A.* 96:10875-10880.
- Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and Alfano, J. 2004. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. *Plant J.* 37:554-565.
- Jin, H., Axtell, M. J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B., and Baker, B. 2002. NPK1, an MEKK1-like mitogen-activated protein kinase, regulates innate immunity and development in plants. *Develop. Cell* 3:291-297.
- Kamoun, S., van West, P., Vleeshouwers, V. G., de Groot, K. E., and Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* 10:1413-1426.
- Keith, R. C., Keith, L. M., Hernandez-Guzman, G., Uppalapati, S. R., and Bender, C. L. 2003. Alginate gene expression by *Pseudomonas syringae* pv. tomato DC3000 in host and non-host plants. *Microbiology* 149(5):1127-1138.
- Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. P. 2002a. Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* 30:415-429.
- Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. P. 2002b. Virus induced gene silencing in tomato. *Plant J.* 31:777-786.
- Lu, R., Malcuit, I., Moffett, P., Ruiz, M. T., Peart, J., Wu, A. J., Rathjen, J. P., Bendahmane, Day, L. and Baulcombe, D. C. 2003. High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:5690-5699.
- Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
- Mudgett, M. B., and Staskawicz, B. J. 1999. Characterization of the *Pseudomonas syringae* pv. tomato AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. *Mol. Microbiol.* 32:927-941.
- Mysore, K. S., and Ryu, C. M. 2004. Nonhost resistance: how much do we know? *Trends Plant Sci.* 9:97-104.
- Peart, J. R., Cook, G., Feys, B. J., Parker, J. E., and Baulcombe, D. C. 2002a. An *EDS1* orthologue is required for N-mediated resistance against tobacco mosaic virus. *Plant J.* 29:569-579.
- Peart, J. R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D. C., Schausser, L., Jaggard, D. A. W., Xiao, S., Coleman, M. J., Dow, M., Jones, J. D., Shirasu, K., and Baulcombe, D. C. 2002b. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. *Proc. Natl. Acad. Sci. U.S.A.* 99:10865-10869.
- Petnicki-Ocwieja, T., Schneider, D. J., Tam, V. C., Chancey, S. T., Shan, L., Jamir, Y., Schechter, L. M., Janes, M. D., Buell, C. R., Tang, X., Collmer, A., and Alfano, J. R. 2002. Genome wide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. tomato DC3000. *Proc. Natl. Acad. Sci. U.S.A.* 99:7652-7657.
- Rathjen, J. P., Chang, J. H., Staskawicz, B. J., and Michelmore, R. W. 1999. Constitutively active *Pto* induces a *Prf*-dependent hypersensitive response in the absence of *avrPto*. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:3232-3240.
- Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. 1992. The cloned avirulence gene *AvrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J. Bacteriol.* 174:1604-1611.
- Salmeron, J. M., Oldroyd, G. E. D., Rommens, C. M. T., Scofield, S. R., Kim, H. S., Lavelle, D. T., Dahlbeck, D., and Staskawicz, B. J. 1996. Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* 86:123-133.
- Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* 274:2063-2065.
- Sessa, G., and Martin, G. B. 2000. Signal recognition and transduction mediated by the tomato *Pto* kinase: a paradigm of innate immunity in plants. *Microbes Infect.* 2:1591-1597.
- Shan, L., He, P., Zhou, J. M., and Tang, X. 2000a. A cluster of mutations disrupt the avirulence but not the virulence function of *AvrPto*. *Mol. Plant-Microbe Interact.* 6:592-598.
- Shan, L., Venkatappa, K., Martin, G. B., Zhou, J. M., and Tang, X. 2000b. The *Pseudomonas* *AvrPto* protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell* 12:2323-2337.
- Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. 1996. Initiation of plant disease resistance by physical interaction of *AvrPto* and *Pto* kinase. *Science* 274:2060-2063.
- Tang, X., Xie, M., Kim, Y. J., Zhou, J., Klessig, D. F., and Martin, G. B. 1999. Overexpression of *Pto* activates defense responses and confers broad resistance. *Plant Cell* 11:15-29.
- Thilmony, R. L., Chen, Z., Bressan, R. A., and Martin, G. B. 1995. Expression of the tomato *Pto* gene in tobacco enhances resistance to *Pseudomonas syringae* pv. tabaci expressing *avrPto*. *Plant Cell* 7:1529-1536.
- Thomas, C. M., Tang, S. J., Hammond-Kosack, K., and Jones, J. D. G. 2000. Comparison of the hypersensitive response induced by the tomato *Cf-4* and *Cf-9* genes in *Nicotiana* spp. *Mol. Plant-Microbe Interact.* 13: 465-469.
- Thordal-Christensen, H. 2003. Fresh insights into processes of nonhost resistance. *Curr. Opin. Plant Biol.* 6: 351-357.
- Wright, C.A. and Beattie, G. A. 2004. *Pseudomonas syringae* pv. tomato cells encounter inhibitory levels of water stress during the hypersensitive response of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 101: 3269-3274.