Intergeneric Transfer and Functional Expression of the Tomato Disease Resistance Gene Pto

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Plant disease resistance loci have been used successfully in breeding programs to transfer traits from resistant germplasm to susceptible plant cultivars. The molecular cloning of plant disease resistance genes now permits the transfer of such traits across species boundaries by genetic transformation of recipient hosts. The tomato disease resistance gene Pto confers resistance to strains of the bacterial pathogen Pseudomonas syringae pv tomato expressing the avirulence gene avrPto. Transformation of Nicotiana benthamiana with Pto results in specific resistance to R. s. pv tabaci strains carrying avrPto. The resistant phenotype is manifested by a strong inhibition of bacterial growth and the ability to exhibit a hypersensitive response. Resistance cosegregates with the Pto gene in transgene selfings and testcrosses. Our results demonstrate the conservation of disease resistance functions across genus boundaries and suggest that the utility of host-specific resistance genes can be extended by intergeneric transfer.

INTRODUCTION

Plants are constantly challenged by phytopathogenic attack. Therefore, plants necessarily express resistance to most pathogens. Disease resistance is often manifested by the hypersensitive response (HR), a rapid localized death of infected plant cells that is thought to limit pathogen growth (Staskawicz et al., 1995). According to the gene-for-gene hypothesis, originally proposed by Flor (1971), HR-correlated disease resistance is specified by the presence of a single dominant or semidominant disease resistance gene in the host and a corresponding avirulence gene in the pathogen. The absence of the avirulence or the resistance gene in either components of the plant–pathogen interaction results in a compatible interaction and leads to plant disease.

Crop plants grown as genetically uniform monocultures are particularly vulnerable to the appearance of virulent races and disease outbreaks that may result in severe yield losses. To control epidemics, commonly used cultivars often have to be replaced within ~3 to 5 years with new cultivars that carry additional loci for resistance (Agrios, 1988; McDermott and McDonald, 1993). The demand for novel disease resistance loci requires a continuous effort by plant breeders to genetically identify and characterize resistant germplasm. Unfortunately, disease resistance loci can often only be transferred to sexually compatible plant cultivars. Another disadvantage of classic breeding programs is that the development of resistant cultivars may take more than a decade. A particular pathogen may threaten crops for many years before a resistant cultivar can be released (Agrios, 1988).

Recently, a number of plant disease resistance genes have been cloned (Martin et al., 1993; Bent et al., 1994; Jones et al., 1994; Mindrinos et al., 1994; Whitham et al., 1994; Ellis et al., 1995). The availability of these genes now permits tests to answer a classic question in phytopathology: Will resistance genes from one species function in other plant species? To address this question, we have focused on the tomato Pto gene. 

Pto confers resistance to strains of Pseudomonas syringae pv tomato expressing the avirulence gene avrPto (Ronald et al., 1992). In the absence of resistance, infection of tomato plants with P. s. tomato results in bacterial speck disease. This disease, which has become economically important throughout the world since the mid-1970s, is characterized by the appearance of small water-soaked lesions surrounded by chlorotic halos (Carland and Staskawicz, 1993). A disease that is similar to bacterial speck disease of tomato is the wildfire disease of Nicotiana spp. Wildfire disease is caused by P. s. pv tabaci and appears as necrotic lesions that eventually become surrounded by large chlorotic halos. The similarity between bacterial speck and wildfire diseases suggests that Nicotiana spp that express susceptibility to P. s. tabaci strains expressing avrPto may be a good model species for testing the function of Pto in a heterologous system.

Both the avrPto gene and the Pto gene have been cloned and characterized. The avrPto gene encodes a mostly hydrophilic protein of 183 kD that has no significant homologies with known protein sequences (Ronald et al., 1992; Salmeron and Staskawicz, 1993). In contrast, the putative...

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product of the disease resistance gene \( Pto \) shares extensive homology with the catalytic domain of many serine–threonine protein kinases (Martin et al., 1993). Subsequent biochemical experiments have demonstrated that the \( Pto \) protein is enzymatically active as a protein kinase (Loh and Martin, 1995; Rommens et al., 1995), indicating its possible involvement in signal transduction. A second component in the \( Pto \)-controlled signaling pathway, which has been identified and genetically characterized, is the \( Prf \) (for \textit{Pseudomonas} resistance and fenitrothion sensitivity) gene (Salmeron et al., 1994). Because disease resistance genes appear to act epistatically as components of signaling pathways, transfer of a particular gene to heterologous plant species may not elicit disease resistance if the recipient host species lacks ancillary proteins that interact with the product of the transferred gene. To determine whether transfer of disease resistance to sexually incompatible hosts can be accomplished by transformation with single cloned resistance genes, we introduced the \( Pto \) gene into \textit{N. benthamiana}.

Our data showed that \( Pto \) function can be transferred across genera and demonstrated that functional components of disease resistance pathways are conserved between different genera.

**RESULTS**

**Response of \textit{Nicotiana} spp to Pathogens Expressing \textit{avrPto}**

Expression of the \( Pto \) gene in tomato results in resistance to strains of \textit{P. s. tomato} carrying the avirulence gene \textit{avrPto}. \textit{N. tabacum} has been shown by DNA gel blot analysis to contain a number of \( Pto \) homologs (Martin et al., 1993). As shown in Figure 1, similar results were obtained for \textit{N. benthamiana} and \textit{N. clevelandii}. These results indicate that several \textit{Nicotiana} spp may contain gene families similar to the tomato \( Pto \) gene family. To study the responses of \textit{N. tabacum}, \textit{N. benthamiana}, and \textit{N. clevelandii} to infection with pathogens expressing \textit{avrPto}, this avirulence gene was introduced into \textit{P. s. pv angulata} and \textit{P. s. tabaci} strains 113 and 11528, respectively. Plants were inoculated by immersion in suspensions containing either the wild-type strains or the transconjugant strains containing \textit{avrPto} and screened after 4 days. In all cases, infection with the wild-type strains resulted in the development of water-soaked disease symptoms (Table 1); strains expressing \textit{avrPto} also caused disease in \textit{N. tabacum} and \textit{N. benthamiana}. However, disease symptoms were not observed on \textit{N. clevelandii} plants infected with strains expressing \textit{avrPto} (Table 1).

More controlled infections were also evaluated by pipette infiltrations of the wild-type and transconjugant strain 11528 at concentrations of \( 10^8 \) colony-forming units (cfu)/mL. Inoculation of \textit{N. benthamiana} in both cases resulted in a water-soaking response. This water soaking subsequently spread beyond the inoculated region and eventually encompassed the entire leaf. Infiltration of \textit{N. tabacum} with both the wild-type and transconjugant strains resulted in a necrotic collapse of the inoculated region within 48 hr. This collapse remained confined to the inoculated region. A similar response was observed in \textit{N. clevelandii}.

**Table 1. Response of \textit{Nicotiana} spp to Infection with Wild-Type \textit{P. syringae} Strains and Transconjugant Strains Expressing \textit{avrPto}**

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>\textit{P. s. tabaci} 11528</th>
<th>\textit{P. s. tabaci} 113</th>
<th>\textit{P. s. angulata}</th>
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<tr>
<td>\textit{N. tabacum} &quot;Turk&quot;</td>
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<td>S</td>
<td>S</td>
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<tr>
<td>\textit{N. tabacum} &quot;Glurk&quot;</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>\textit{N. benthamiana}</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>\textit{N. clevelandii}</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

\( ^a \) Plants were infected by immersion in bacterial suspensions (5 x \( 10^7 \) cfu/mL for \textit{N. benthamiana}; 5 x \( 10^6 \) cfu/mL for \textit{N. tabacum} and \textit{N. clevelandii}) and tested after 4 days for the development of disease symptoms. Plants displaying water-soaked lesions were considered susceptible (S); plants not displaying any disease symptoms were classified as resistant (R). Wild-type strains are indicated with (−); transconjugant strains expressing \textit{avrPto} are indicated with (+).
Expression of Pto in N. benthamiana

inoculated with the wild-type strain. However, inoculation of N. clevelandii with the transconjugant strain resulted in an accelerated plant response: the entire inoculated region necrotized within 14 hr. The latter observation confirms the differential N. clevelandii host response to avrPto noted above and indicates that disease resistance in N. clevelandii is correlated with the induction of an HR. These results suggest that N. clevelandii may contain a functionally active Pto homolog.

To determine whether the susceptible and resistant phenotypes reflected the level of bacterial growth in the plant, growth curve experiments were performed. As shown in Figure 2, wild-type P. s. tabaci 11528 grew to high levels in all plants tested, reaching levels of $5 \times 10^6$ to $5 \times 10^7$ cfu/cm$^2$ of leaf tissue after 4 days. This growth rate is characteristic of a compatible interaction. The presence of avrPto in P. s. tabaci 11528 resulted in slight reductions of growth in N. tabacum and N. benthamiana plants, as has been described previously for susceptible tomato plants inoculated with P. s. tabaci carrying avrPto (Ronald et al., 1992). In N. clevelandii, expression of avrPto in P. s. tabaci resulted in a dramatic $10^3$-fold reduction of bacterial growth (Figure 2); this parallels the kinetics observed in resistant tomato plants (Ronald et al., 1992). These results demonstrate that N. tabacum and N. benthamiana are susceptible to P. s. tabaci strains expressing avrPto and that N. clevelandii displays resistance to this pathogen to a degree similar to that seen in resistant tomato lines.

Figure 2. Growth in Nicotiana spp of Wild-Type P. s. tabaci 11528 and a Transconjugant Expressing avrPto.

Concentration of bacteria in leaves was assayed 0, 2, and 4 days after inoculation. Data points, indicated in log (colony-forming units per square centimeter), represent the mean of three replicate experiments ± SD. Open and closed symbols represent data points for the wild-type strain and the transconjugant strain expressing avrPto, respectively.

Resistance of Transgenic N. benthamiana Plants Expressing Pto to P. s. tabaci Carrying avrPto

To test whether Pto could function in a heterologous host, the Pto gene was stably introduced into N. benthamiana by
Agrobacterium-mediated transformation. We chose this particular species of *Nicotiana* because it does not recognize strains of *P. s. tabaci* carrying the *avrPto* avirulence gene. A binary vector was used that contained the 1.0-kb open reading frame 1 (ORF1) of *Pto* fused to the 35S promoter of cauliflower mosaic virus between the borders of the T-DNA (Figure 3). *Pto* ORF1 had been shown previously to be functional in conferring resistance to pathogens expressing *avrPto* in tomato (Martin et al., 1993; Rommens et al., 1995). Five primary transformants were analyzed for expression of *Pto* by isolating total RNA and performing polymerase chain reaction (PCR) on reverse-transcribed RNA using *Pto*-specific primers. As shown in Figure 4, products with an expected length of 800 bp could be amplified in four of five transformants. Regenerated shoots of the transformants expressing *Pto* were immersed in suspensions of either the wild-type strain 11528 or strain 11528 carrying *avrPto* and screened for the development of disease symptoms. The four transformants were fully susceptible to wild-type *P. s. tabaci* 11528 (results not shown).

However, disease symptoms failed to develop in any of the *Pto*-expressing plants after infection with strains expressing *avrPto* (Figure 5). Resistance to *P. s. tabaci* 11528 expressing *avrPto* was also correlated with the ability of the *Pto* transformants to develop a rapid visible HR when inoculated with high concentrations (10⁸ cfu/mL) of *P. s. tabaci* expressing *avrPto*. In these resistant plants, the inoculated tissue collapsed and the entire inoculated region became necrotic 16 hr after pipette inoculation (Figure 5). In contrast, inoculation of untransformed *N. benthamiana* plants with wild-type strain 11528 resulted in water-soaked disease symptoms after 22 hr (Figure 5).

To confirm that disease resistance was due to *Pto* gene expression, two of the transformed plants expressing *Pto*, designated 38-12 and 38-9, were self-fertilized and backcrossed to wild-type *N. benthamiana* plants. As shown in Table 2, PCR analysis of progeny plants of transformant 38-12 showed that the *Pto* gene was inherited by 16 of 23 progeny of selfed plants and 11 of 26 progeny of testcrossed plants. These results are consistent with segregation ratios of 3:1 and 1:1, respectively, and suggest the presence of a single *Pto* locus in the original transformant. Progeny plants were inoculated with *P. s. tabaci* expressing *avrPto* and scored after 4 days. Strict cosegregation was observed for *Pto* and disease resistance, indicating that the *Pto* gene confers disease resistance when expressed in transgenic *N. benthamiana*. The segregation ratios in offspring of transformant 38-9 were not compatible with Mendelian ratios and resembled the unexplained aberrant ratios observed previously for transgenic *N. benthamiana* lines (Rubino et al., 1993). Nevertheless, the inoculation data still demonstrated a strict cosegregation of the *Pto* gene with resistance to *P. s. tabaci* expressing *avrPto* (Table 2). As a control, 4 weeks after infection of progeny plants with *P. s. tabaci* 11528 expressing *avrPto*, resistant progeny plants were infected with wild-type strain 11528. All plants developed severe disease symptoms,

![Figure 3](image-url)  
**Figure 3.** The T-DNA Region of SLJ44024 Carrying *Pto ORF1*. The 8.4-kb region between the left border (LB) and the right border (RB) is indicated. The T-DNA region contains a 1.0-kb *Pto* fragment between the 35S promoter of cauliflower mosaic virus (p-35S) and the terminator of the nopaline synthase gene (t-nos). The cloned *Pto* fragment corresponds to ORF1 of a 2.4-kb *Pto* cDNA isolated by Martin et al. (1993). The presence of the neomycin phosphotransferase gene (Wpt), driven by the promoter of the nopaline synthase gene (p-nos), allows selection of transformed plants for kanamycin resistance.

![Figure 4](image-url)  
**Figure 4.** Detection of *Pto* Transcripts in Transgenic *N. benthamiana* Plants by Reverse Transcription–PCR. Reactions were performed in the presence (even lanes) and absence (odd lanes) of reverse transcriptase. RNAs used were isolated from five independently transformed plants (lanes 1 to 10; two lanes per plant) and an untransformed plant (lanes 11 and 12). Based on the positions of the DNA markers (Gibco BRL) in lane 13, the estimated length of the reverse transcription–PCR products in lanes 4, 6, 8, and 10 is 0.8 kb.
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Figure 5. Resistance of Transgenic N. benthamiana Plants Expressing Pto to P. s. tabaci Carrying avrPto.

(A) to (D) Leaves of Pto-transformed ([A] and [C]) and wild-type ([B] and [D]) plants were immersed in a suspension containing $5 \times 10^7$ cfu/mL of the transconjugant strain 11528 carrying avrPto and photographed after both 4 days ([A] and [B]) and 3 weeks ([C] and [D]). The development of water-soaked lesions and wildfire symptoms, respectively, are indicative of a compatible interaction.

(E) and (F) Leaves of Pto-transformed (E) and wild-type (F) plants were also infected by pipette infiltration of both the avrPfo-expressing strain (left side of leaf) and the wild-type strain (right side of leaf) at concentrations of $10^8$ cfu/mL and photographed after 2 days. A dark water-soaking reaction is indicative of a compatible interaction; the development of necrosis indicates an incompatible interaction. At this level of pathogen inoculation, the water-soaking response includes pathogen-induced necrosis; however, this is very different from the pale brown necrosis indicative of an HR.

demonstrating that Pto-based resistance in N. benthamiana is dependent on the presence of avrPto in P. s. tabaci.

To quantify the effects of Pto gene expression on the growth of the wild-type or transconjugant P. s. tabaci, bacterial growth was monitored in resistant progeny plants derived from test-crosses of transgenic plants. As illustrated in Figure 6, the virulent wild-type strain grew to high levels in both transgenic plants tested. As expected, expression of avrPto strongly inhibited growth of P. s. tabaci in progeny plants carrying Pto, resulting in a 500-fold reduction of bacterial growth (Figure 6). We conclude that the avrPto–Pto interaction, which specifies resistance in tomato to P. s. tomato, can be engineered to provide effective resistance in N. benthamiana plants against P. s. tabaci strains carrying avrPto.

DISCUSSION

Plants expressing disease resistance loci have been used extensively in conventional breeding programs to introgress resistance into sexually compatible plants. In this study, we demonstrate that a disease resistance gene isolated from the tomato Pto locus is functional in N. benthamiana. Our data further demonstrate that resistance genes can be functional in plant species that belong to different genera and suggest that cloned resistance genes may be transferred across genera.

Resistance of transgenic N. benthamiana plants expressing Pto implies that these plants contain functional homologs of all other components of the tomato Pto-controlled pathways that are essential for development of the hypersensitive disease resistance response. Currently, this pathway is also known to involve the Prf gene (Salmeron et al., 1994). This locus has been genetically identified and characterized and is thought to be an essential intermediate in the signal transduction pathway for both resistance to the pathogen and sensitivity to the insecticide fenthion. These results also suggest that signal transduction pathways involved in disease resistance are conserved within some members of the Solanaceae. It will be interesting to determine the function of Pto in plant species that are classified in other families, such as Arabidopsis and Chenopodium amaranticolor. These species are susceptible to P. s. tomato DC3000 (which contains avrPto) and P. s.

<table>
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<th>Parent</th>
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<td></td>
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<tr>
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<td>0</td>
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<td>Transformant 38-9</td>
<td>8</td>
<td>0</td>
<td>1:1 (3.84)</td>
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</table>

a (+) N. benthamiana containing Pto; (-) wild-type N. benthamiana.

b R, resistant.
c S, susceptible.
d Numbers in parentheses indicate chi square. A x² of 3.84 is significant at the 0.05 probability level.
e NA, not applicable.
Which the avirulence gene was obtained. Similar results have
provided no novel modes of resistance. For Po to function,
which avirulence gene to bacteria expressing
avrPto is structurally different from the tomato Po gene.
Resistance to P. s. tomato expressing avrPto cosegregates
with sensitivity to the organophosphate fenthion (Laterrot and
Philouze, 1985). This association can be ascribed to either
pleiotropy or tight linkage of two distinct genes. Arguments for
the latter hypothesis are (1) that mutants of resistant tomato
lines that are now susceptible to avrPto-expressing bacteria
retain fenthion sensitivity (Salmeron et al., 1994), and (2) that
fenthion sensitivity in tomato plants lacking Po can be con-
ferred by the Fen gene, which is distinct from Po (Martin et
al., 1994; Rommens et al., 1995). Because the involvement
of Po in fenthion sensitivity cannot be excluded from these
studies, we immersed transgenic N. benthamiana plants
expressing Pto into a solution containing fenthion (C.M.T.
Rommens and B.J. Staskawicz, unpublished results). Necrotic
specks, indicative of fenthion sensitivity, failed to develop in
these plants. These results support the argument that the Po
gene functions specifically in pathogen recognition in tomato.
Applications of genetic engineering for the transfer of dis-
ease resistance between sexually incompatible species hold
much promise for the future. The ability to transfer disease
resistance genes between taxonomically diverse plants will
provide answers to many basic questions concerning the mech-
nisms of how plants recognize pathogens and how they are
able to express resistance to the majority of pathogens that
they encounter. This information can then be employed in
strategies to develop durable disease resistance in agronom-
ically important crop species.

METHODS

Plant Materials, Bacterial Strains, Infections, and Growth
Curves

Tobacco (Nicotiana spp) plants were grown in clay pots in standard
potting soil. Strains used were Pseudomonas syringae pv tabaci 11528
and P. s. pv angulata 113. The pDSK519-derived plasmid pTiOC38,
carrying the avirulence gene avrPto (Ronald et al., 1992), was intro-
duced into P. syringae strains by triparental mating using the helper
strain pRK2013 (Figurski and Helinski, 1979). To assay for susceptibil-
ity or resistance to P. s. tabaci, plants were inoculated with 5 x 10^7
colony-forming units (cfu)/mL of the pathogen and
0.03% Silwet L77 (Osi Specialties, Sisterville, WV), inoculated by pi-
ette infiltration at concentrations of either 10^8 or 10^12 cfu/mL (Whalen
et al., 1988; Kunkel et al., 1993), or vacuum infiltrated with 5 x 10^6
cfu/mL of bacteria for analysis of bacterial growth, as described by
Carland and Staskawicz (1993). Fenthion treatments were also per-
formed as described by Carland and Staskawicz (1993).
Polymerase Chain Reaction and Primers

Polymerase chain reactions (PCR) were performed according to Klimyuk et al. (1992). Pto-specific primers were used to amplify the 0.5-kb 3' part of the Pto gene were 5'-AGAGGCTGGGATATGCAATG-3' and 5'-GCCATATCTCAGAACTATATAAACCTCGAAGAAC-3'. To determine whether transformed plants expressed Pto, total RNA from these plants was analyzed by reverse transcription–PCR according to Simpson et al. (1992). Primers used for this purpose were 5'-TCGATATCGATGGGATACG-3' (5' primer) and 5'-TCTGCAAGATTGGGATCTACG-3' (3' primer).

Plasmid Construction and Agrobacterium tumefaciens-Mediated Transformation

The Pto gene was isolated as a 1.0-kb ClaI-XbaI fragment from pBluePto (Rommens et al., 1992) and cloned into the corresponding sites of the binary plasmid SLJ44024 (Jones et al., 1992). The binary vector carrying Pto was conjugated into the Agrobacterium strain LBA4404 by triparental mating. N. benthamiana plants were transformed as described by McCormick et al. (1986).

DNA Gel Blot Analysis

DNAs from the resistant tomato line 76R (Pto PrfPto Prf) and Nicotiana spp were isolated as previously described (Carland and Staskawicz, 1993). DNA gel blot analysis was performed according to protocols supplied with Hybond N+ membrane (Amersham).

ACKNOWLEDGMENTS

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