The Pseudomonas AvrPto Protein Is Differentially Recognized by Tomato and Tobacco and Is Localized to the Plant Plasma Membrane

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The avrPto gene of Pseudomonas syringae pv tomato triggers race-specific resistance in tomato plants carrying Pto, a resistance gene encoding a protein kinase. When introduced into P. s. tabaci, avrPto triggers resistance in tobacco W38 plants that carry the corresponding R gene. The AvrPto protein is believed to be secreted into host cells through the bacterial type III secretion pathway, where it activates disease resistance in tomato by interacting with Pto. We report here the identification of two distinct regions in AvrPto that determine the recognition specificity of this protein in tomato and tobacco. Point mutations in the central region disrupted the avirulence activity in tomato but not in tobacco. Conversely, point mutations in the C-terminal region abolished the avirulence in tobacco but not in tomato. We further report that AvrPto was localized to the plasma membrane of plant cells. Disrupting the membrane association by mutating a putative myristoylation motif of AvrPto abolished the avirulence activity in both tomato and tobacco. These findings demonstrate that AvrPto is recognized differently by the R genes in tomato and tobacco and that the recognition of AvrPto probably is associated with the plasma membrane.

INTRODUCTION

Avirulence (avr) genes trigger gene-for-gene resistance in plants containing the corresponding disease resistance (R) genes (Staskawicz et al., 1995). Numerous avr genes have been cloned from various bacterial pathogens. Recent studies suggest that bacterial Avr proteins are secreted into the host cells through the type III secretion pathway and subsequently are perceived by the corresponding R gene products (reviewed in Alfano and Collmer, 1997; Lindgren, 1997; He, 1998; Kjemtrup et al., 2000).

The AvrBs3 protein represents one family of bacterial Avr proteins that share high sequence similarities. These proteins contain a variable number of nearly identical repeat domains in the internal portion of the protein (Leach and White, 1996). Deletion and mutagenesis analysis indicate that the internal repeat region determines the recognition specificity of the AvrBs3 protein (Herbers et al., 1992). AvrBs3-like proteins contain a nuclear localization sequence and have been detected in plant nuclei (Yang and Gabriel, 1995; Van den Ackerveken et al., 1996; Zhu et al., 1998, 1999). The transcription activity of AvrXa10, a member of the AvrBs3 family, in eukaryotic cells is essential for its avirulence function (Zhu et al., 1999). The AvrBs3-like proteins may mediate the avirulence function by regulating plant gene expression.

Other bacterial Avr proteins do not carry a characteristic sequence indicative of recognition specificity and in planta localization (Leach and White, 1996). The reciprocal interchanges between AvrB and AvrC, two Avr proteins that share considerable sequence similarity, have identified a large central region in AvrB and AvrC that determines the recognition specificity (Tamaki et al., 1991). The rest of the bacterial Avr proteins do not share much homology with each other or with other known proteins (Leach and White, 1996). Characterization of the specificity determinants of these proteins has been difficult because the sequences of these proteins provide no clue to what region might be recognized by the R proteins. Consequently, the sequences of these proteins that determine the recognition specificity remain completely unknown. Three Avr proteins in this group recently were reported to be associated with the plant plasma membrane (Nimchuk et al., 2000); where the other Avr proteins in this group are localized in plant cells is not known.

The AvrPto protein of Pseudomonas syringae pv tomato provides an excellent model system for the structure–function study of bacterial avirulence proteins. AvrPto confers disease resistance in tomato plants containing Pto, an R...
gene that encodes a serine/threonine protein kinase (Ronald et al., 1992; Martin et al., 1993). Transient expression of AvrPto in plant cells bypasses the requirement of pathogens and induces a Pto-dependent hypersensitive response (HR), suggesting that the AvrPto protein acts inside the plant cell (Gopalan et al., 1996; Scofield et al., 1996; Tang et al., 1996). In the yeast two-hybrid system, AvrPto interacts physically with Pto, an interaction that determines the Pto–avrPto recognition specificity (Scofield et al., 1996; Tang et al., 1996). When AvrPto is expressed in P. s. tabaci, it triggers the HR and disease resistance in tobacco W38 plants carrying an R gene that has not been cloned (Thilmony et al., 1995).

To understand how AvrPto confers recognition specificity and where in the plant cell AvrPto is recognized by the resistance genes, we performed mutagenesis and protein localization studies with AvrPto. Here, we report the identification of two distinct regions of AvrPto that determine its specific recognition by the R genes in tomato and tobacco plants. The region that specifies recognition by tomato Pto is localized in the middle of AvrPto, whereas the region that determines specific recognition by the tobacco resistance gene resides at the C terminus of the AvrPto protein. In addition, we found that AvrPto is associated with the plant plasma membrane: mutation of a putative myristoylation site at the N terminus of AvrPto disrupted the membrane localization of AvrPto and completely abolished the avirulence activity in both tomato and tobacco plants. These results demonstrate that the cognate tomato and tobacco resistance genes differentially recognize a plasma membrane–located AvrPto protein.

RESULTS

Generation and Identification of AvrPto Mutants That Do Not Interact with Pto

AvrPto interacts physically with Pto in the yeast two-hybrid system, and this interaction determines the Pto–avrPto recognition specificity (Scofield et al., 1996; Tang et al., 1996). To define the sequence of AvrPto that is required for Pto recognition, we developed a reverse yeast two-hybrid protocol to identify point mutations in AvrPto that disrupt the interaction with Pto.

We first generated a mutant avrPto library by polymerase chain reaction (PCR) amplification of the avrPto gene. The avrPto mutant library (in the prey plasmid pJG4-5; Golemis et al., 1997) was transformed into a yeast strain expressing Pto as the bait protein. The interaction of wild-type AvrPto and Pto activated the reporter gene LacZ, giving rise to dark blue yeast colonies on a plate containing X-Gal. AvrPto mutants that were unable to interact with Pto failed to activate the LacZ gene, and the yeast colonies were white. From 4000 yeast colonies, we identified 49 AvrPto mutants that disrupted the Pto interaction, as indicated by the lack of LacZ gene expression in the plate assay. Sequence analysis of the 49 AvrPto mutants revealed 40 unique mutants and nine siblings. Among the 40 mutants, 10 carried single amino acid substitutions and the rest contained multiple mutations, frameshifts, or an early stop codon. The mutations were distributed throughout the AvrPto protein, indicating that the mutagenesis was random and efficient.

In the 10 mutants with single amino acid substitutions, we examined whether the lack of LacZ gene expression resulted from the disruption of protein–protein interaction or the instability of the AvrPto protein in yeast. Protein gel blot analysis showed that seven mutants (L43P, L72P, Q86R, S94P, I96T, G99V, and N105K) were expressed in yeast at approximately the same amount as the wild-type AvrPto, whereas two mutants (M11T and S46P) exhibited small amounts of protein. These mutations are summarized in Table 1. One mutant produced a protein smaller than the wild-type AvrPto and was not studied further.

AvrPto Mutations Disrupting Pto Interaction Abolish the Avirulence Activity in Tomato

To study the effect of the single amino acid substitutions on the avirulence activity of AvrPto in tomato plants, we introduced the nine avrPto mutants described in Table 1 into the P. s. tomato T1 strain and inoculated each of the resulting strains into Rio Grande–PtoR plants (carrying the Pto gene). The bacterial numbers of T1 strains carrying avrPto mutants were compared with those of T1 and T1(avnPto) (Figure 1). Four days after inoculation, T1(avnPto) multiplied <0.002 as much as T1 did, as a result of the Pto-mediated resistance. In contrast, the bacterial numbers of all of the strains carry-

| Table 1. Characterization of AvrPto Mutants Disrupting the Pto Interaction |
|-----------------------------|-----------------|------------------|
| Mutant | Pto Interaction | Protein Accumulation |
| AvrPto | Yes | Normal |
| M11T | No | Low |
| L43P | No | Normal |
| S46P | No | Low |
| L72P | No | Normal |
| Q86R | No | Normal |
| S94P | No | Normal |
| I96T | No | Normal |
| G99V | No | Normal |
| N105K | No | Normal |

*Mutants were named according to their amino acid substitutions. Single-letter code is used to designate the amino acids. 
*Pto interaction was determined by yeast two-hybrid assay. 
*Mutant proteins were examined by protein gel blot analysis using the anti-AvrPto antibodies.
AvrPto-specific resistance protein, which was present only in the bacterial cells (Figure 3B), indicating that these mutations did not disrupt protein secretion.

We also examined two representative AvrPto mutant proteins, S46P (representing the unstable AvrPto mutants in Pseudomonas) and S94P (representing the relatively stable AvrPto mutants), that were expressed directly in tomato and tobacco plants by using Agrobacterium-mediated gene expression (Tang et al., 1996) and the tetracycline-inducible gene expression system (Gatz et al., 1992). Although abundant protein was detected for S94P, S46P was barely detectable in plants (data not shown), suggesting that the stability of AvrPto mutants in Pseudomonas is correlated with their stability in plants. Unlike AvrRpt2 and AvrPphB proteins (Mudgett and Staskawicz, 1999; Nimchuk et al., 2000), no internal cleavage was detected for AvrPto or its derivative proteins in bacteria or in planta. The abundant protein (Mudgett and Staskawicz, 1999; Nimchuk et al., 2000), no internal cleavage was detected for AvrPto or its derivative proteins in bacteria or in planta. The abundant protein expression (Tang et al., 1996) and the tetracycline-inducible gene expression system (Gatz et al., 1992). Although abundant protein was detected for S94P, S46P was barely detectable in plants (data not shown), suggesting that the stability of AvrPto mutants in Pseudomonas is correlated with their stability in plants. Unlike AvrRpt2 and AvrPphB proteins (Mudgett and Staskawicz, 1999; Nimchuk et al., 2000), no internal cleavage was detected for AvrPto or its derivative proteins in bacteria or in planta. The abundant amounts of S94P, I96T, and G99V proteins were consistent with the full avirulence activity of these three mutants in tobacco W38 plants, which suggests that the loss of avirulence activity in tomato plants probably resulted from the disruption of Pto–AvrPto recognition.

The amino acid residues defined by mutants S94P, I96T, and G99V were similar to or greater than those of T1, indicating a complete loss of avirulence activity. The bacterial numbers in PtoR tomato plants were closely correlated with the disease symptoms caused by these strains (data not shown).

Mutants S94P, I96T, and G99V Display the Avirulence Function in Tobacco

Tobacco cultivar W38 plants are resistant to P. s. tabaci carrying the avrPto gene (Thilmony et al., 1995), a resistance that is determined by a single, dominant gene (X. Tang, unpublished results). To test how the tobacco R gene reacts to the nine avrPto mutants described above, we introduced the mutants into P. s. tabaci and inoculated the strains onto W38 plants. Six strains carrying avrPto mutants (M11T, L43P, S46P, L72P, Q66R, and M105K) produced water-soaked lesions on tobacco plants identical to those caused by P. s. tabaci without the avrPto gene. Surprisingly, however, mutants S94P, I96T, and G99V gave resistance identical to that conferred by the wild-type avrPto gene (Figure 2A) and also induced an HR indistinguishable from that caused by the wild-type avrPto (Figure 2B). To quantitatively determine the resistance conferred by mutants S94P, I96T, and G99V, we compared the bacterial numbers of P. s. tabaci strains carrying these mutants with those of strains carrying the wild-type avrPto, the avrPto mutant N105K, or no avrPto (Figure 2C). Strains carrying mutants S94P, I96T, and G99V showed no discernible difference from P. s. tabaci(avrPto). In contrast, the strain with mutant N105K grew much more than P. s. tabaci(avrPto) and was similar to P. s. tabaci without avrPto. The results indicated that mutants S94P, I96T, and G99V were completely avirulent on tobacco W38 plants, in contrast with the lack of avirulence activity on tomato PtoR plants.

The stability and secretion of the AvrPto mutant proteins were studied to understand the differential avirulence activity of AvrPto mutants in tomato and tobacco plants. Figure 3A shows the AvrPto mutant proteins in P. s. tomato. Similar results were obtained with P. s. tabaci (data not shown). Six mutations (M11T, L43P, S46P, L72P, Q66R, and M105K) severely decreased the AvrPto protein stability in Pseudomonas bacteria, suggesting that the protein instability probably accounted for the loss of avirulence activity in these mutants in tomato and tobacco. The mutant I96T protein accumulated normally in Pseudomonas and in the minimal medium. The mutant S94P and G99V proteins were less abundant than AvrPto, but they still accumulated to a substantial amount in both the bacterial cells and the minimal medium. This was different from the periplasmic kanamycin-resistance protein, which was present only in the bacterial cells (Figure 3B), indicating that these mutations did not disrupt protein secretion.
residues. Because mutants S94P, I96T, and G99V specifically disrupted the Pto interaction and the avirulence activity in tomato but maintained the avirulence in tobacco, these mutations probably define a region in AvrPto that is recognized specifically by the tomato Pto protein.

Isolation of AvrPto Mutants that Recognize Pto but Not the Tobacco R Gene

The differential avirulence activity of AvrPto mutants S94P, I96T, and G99V in tomato and tobacco plants indicated that the R genes in the two plant species possess different recognition specificities. This prompted us to study whether distinct regions of AvrPto determine the recognition specificity by the tomato and tobacco resistance genes. We approached this question by isolating AvrPto mutants that lost avirulence activity in tobacco W38 plants but maintained the recognition with tomato Pto.

We constructed an avrPto mutant library in the broad-host-range plasmid pDSK519 (Keen et al., 1988) and introduced the library into P. s. tabaci. Individual clones were tested for the ability to induce the HR in wild-type tobacco W38 plants and in transgenic tobacco W38(35S::Pto) plants carrying the tomato Pto gene under the control of the 35S promoter (Thilmony et al., 1995). AvrPto mutants that maintained the recognition by Pto but disrupted the recognition by the tobacco R gene were expected to cause the HR in W38(35S::Pto) but not in W38 plants. Of the 2000 clones screened, 65 clones did not induce the HR in either W38 or W38(35S::Pto) plants. Eight clones induced normal HR in W38(35S::Pto) but not in W38 plants (Figure 4A). Repeated inoculations with the eight clones gave identical results. We further examined the ability of the eight clones to induce disease resistance in W38(35S::Pto) plants. Figure 4B shows that only five clones (93, 160, 332, 445, and 1603) exhibited marked resistance in W38(35S::Pto) plants, as measured by the development of disease symptoms. These five clones were equally or slightly more virulent than was P. s. tabaci without avrPto when assayed in W38 plants (Figures 4B and 4C), indicating that these mutants had completely lost avirulence activity in W38. The mutant AvrPto proteins are stable in P. s. tabaci and in planta (data not shown). Clones 1686, 1917, and 1938 displayed no particular resistance in W38(35S::Pto) plants (Figure 4B) and were not investigated further.

**Figure 2.** Some AvrPto Mutants Disrupt Avirulence in Tomato but Not in Tobacco.

The AvrPto mutants described in Table 1 were introduced into P. s. tabaci strain 11528R, and the resulting strains were injected separately into tobacco W38 leaves. Inoculum of 10⁶ cfu/mL was used for the HR assay. Inoculum of 10⁸ cfu/mL was used to assay for disease symptoms and to measure bacterial growth in tobacco. (A) Disease symptoms in W38 plants caused by strain 11528R carrying avrPto mutants. Inoculated leaves were photographed 4 days after inoculation. (B) The HR in W38 plant caused by strain 11528R carrying avrPto mutants. Inoculated leaves were photographed 18 hr after inoculation. (C) Growth of strain 11528R carrying avrPto mutants in W38 plants. Error bars indicate SE.
To determine whether these five avrPto mutants conferred resistance in tomato PtoR plants, we introduced them into P. s. tomato T1 strain and inoculated each strain (at 10^4 colony-forming units [cfu]/mL) into PtoR plants. In contrast with the lack of avirulence activity in W38 plants, T1 strains carrying the five avrPto mutants multiplied much less than did the T1 strain without the avrPto gene (Figure 4D). The low bacterial numbers correlated with the lack of disease symptoms in PtoR plants (data not shown).

AvrPto C Terminus Is Required for Avirulence in Tobacco

Sequencing analysis showed that mutants 160, 332, and 1603 carried single nucleotide changes that resulted in single amino acid substitutions (Table 2). Mutants 93 and 445 carried two nucleotide changes that caused the substitution of two amino acid residues in each protein. Strikingly, the mutations in mutants 160 (S153P), 332 (S147R), and 1603 (P146L) are confined to a region spanning eight amino acid residues near the C terminus of AvrPto. Mutants 93 and 445 also carried mutations in this region (G138K and P146L in mutant 93 and P18Q and N145K in mutant 445). In contrast, 10 randomly selected mutants that disrupted the HR-inducing ability in W38 plants, T1 strains carrying the two deletion mutants were 10- to 20-fold more than those of the strain carrying the wild-type avrPto but were <1% of those of the strain without avrPto (Figure 5A). These findings suggest that the C terminus of AvrPto is essential for the avirulence activity in tobacco but only quantitatively affects the avirulence activity in tomato. Deletion of AvrPto as far as 25 amino acids from the C terminus did not affect the interaction with the tomato Pto proteins (Tang et al., 1996). This implied that the five AvrPto C-terminal mutants described in Table 2, which all carried mutations in this region, might interact with Pto. We constructed these mutants in the prey plasmid, tested the interaction of each mutant protein with the tomato Pto in yeast, and confirmed that the mutants indeed interacted normally with the tomato Pto protein (data not shown).

AvrPto Is Associated with the Plant Plasma Membrane

AvrPto has been suggested to be a cytoplasmic protein because no potential signal peptide or transmembrane domains are found in its sequence (Salmeron and Staskawicz, 1993). Consistent with this fact, the sequence of the Pto kinase also predicts a cytoplasmic localization (Martin et al., 1993). To determine whether the interaction of Pto and AvrPto occurs in the cytoplasm, we investigated the localization of AvrPto in plant cells. We did not examine AvrPto in P. s. tomato–infected plants because of the difficulty of distinguishing the protein in plant...
cells from the protein in bacterial cells. Instead, we used the tetracycline-inducible gene expression system (Gatz et al., 1992) to express the AvrPto protein in plant cells. The tetracycline-inducible system consists of two transgene constructs: one carries the bacterial tetracycline repressor (TetR) under the control of the 35S promoter, and the other carries the gene of interest under the control of a modified 35S promoter that contains the TetR binding sequence adjacent to the TATA box. Transgenic plants carrying both transgenes express a large amount of TetR protein. Consequently, the gene of interest is repressed when the TetR binds to the binding sequence near the TATA box. When

Figure 4. Some AvrPto Mutants Recognize Pto but Not the Tobacco R Gene.

(A) HR induction on W38 and W38(35S::Pto) plants by P. s. tabaci strain 11528R carrying avrPto mutants.
(B) Disease symptoms in W38 and W38(35S::Pto) plants caused by P. s. tabaci strain 11528R carrying avrPto mutants.
(C) Growth of P. s. tabaci strain 11528R carrying avrPto mutants in tobacco W38 plants.
(D) Growth of P. s. tabaci strain T1 carrying avrPto mutants in tomato PtoR plants.

Plant inoculation and bacterial measurement were as described in Figures 1 and 2. Error bars indicate SE.
treated with tetracycline, the TetR protein dissociates from the binding sequence in the promoter, and the gene of interest is expressed.

We generated 52 tobacco W38 plants carrying the *avrPto* gene under the tetracycline-inducible promoter. Of these, 28 plants showed a strong HR 16 to 18 hr after tetracycline infiltration into the leaves (Figure 6A). In contrast, plants transformed with the empty vector failed to show any response to tetracycline application.

We used affinity-purified anti-AvrPto antibodies to detect the AvrPto protein in transgenic W38 plants. Total protein was extracted from tetracycline-treated transgenic tobacco plants (10 hr after treatment) that contained either the *avrPto* transgene or an empty vector. The anti-AvrPto antibodies detected a strong band of ~20 kD from the tetracycline-induced plants carrying the *avrPto* transgene or an empty vector. The anti-AvrPto antibodies were analyzed for the presence of the AvrPto protein. In Figure 6A, a band at 20 kD for the AvrPto protein was detected only in the membrane fraction and was completely absent in the soluble fraction in tetracycline-treated transgenic plants (Figure 6B), indicating that AvrPto was associated exclusively with membrane.

To determine whether the AvrPto protein is localized to the cytoplasm, we separated the total protein into membrane and soluble fractions by centrifugation. Both fractions were analyzed for the presence of the AvrPto protein. In contrast with the predicted cytoplasmic localization, AvrPto was detected only in the membrane fraction and was completely absent in the soluble fraction in tetracycline-treated *avrPto* transgenic plants (Figure 6B), indicating that AvrPto was associated exclusively with membrane.

The total membrane was further separated into plasma membrane and intracellular membrane by means of an aqueous polymer two-phase system (Larsson et al., 1987; Xu et al., 1996). The purity of the two membrane fractions was determined by the distribution of marker enzymes; vanadate-sensitive ATPase for plasma membrane, and NADH-cytochrome c reductase and cytochrome c oxidase for intracellular membrane (Briskin et al., 1987). Most of the vanadate-sensitive ATPase activity was detected in the plasma membrane, whereas the NADH-cytochrome c reductase and cytochrome c oxidase activities were found mostly in the intracellular membrane (Figure 6C, bottom), indicating that the two membrane fractions were well separated. Protein gel blot analysis indicated that AvrPto was associated nearly exclusively with the plasma membrane fraction (Figure 6C, top).

### Table 2. AvrPto Mutations Conferring Disease Resistance in W38(35S::Pto) but Not in Tobacco W38 Plants

<table>
<thead>
<tr>
<th>AvrPto Mutant</th>
<th>Amino Acid Substitutions</th>
<th>W38</th>
<th>W38(35S::Pto)</th>
</tr>
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<tbody>
<tr>
<td>93</td>
<td>E138K, P146L</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>160</td>
<td>S153P</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>332</td>
<td>S147R</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>445</td>
<td>P18Q, N145K</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>1603</td>
<td>P146L</td>
<td>No</td>
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</tr>
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</table>

*a* AvrPto mutants that conferred disease resistance in W38(35S::Pto) but not in W38 were PCR-amplified and sequenced. 
b Mutations indicate the amino acid substitutions. Single-letter code is used to designate the amino acids.

**Mutation of a Putative Myristoylation Site Disrupts the Membrane Association and the Avirulence Function of AvrPto**

To determine the functional relevance of the plasma membrane localization of AvrPto, we sought to disrupt the membrane association by mutagenesis. Examination of the protein sequence revealed a putative myristoylation motif (Met-Gly-X-X-Cys) at the N terminus of AvrPto. Covalent attachment of myristate to the Gly residue is an important mechanism that anchors many proteins to eukaryotic cell membranes (Resh, 1999). We replaced the Gly2 residue with Ala and expressed the mutant protein in tobacco plants by using the tetracycline-inducible gene expression system. The localization of the G2A mutant protein was examined by means of protein fractionation and protein gel blot analysis. As shown in Figure 7A, two bands specific to the G2A-expressing plants, one slightly smaller than the wild-type AvrPto and the other slightly larger than AvrPto, were detected in the soluble fraction but not in the membrane fraction. The larger band is probably the G2A protein, whereas the smaller band may be a degradation product. The results indicated that the putative myristoylation motif in AvrPto is required for membrane association.

We introduced the G2A mutant into the *P. s. tomato* and *P. s. tabaci* strains and examined the avirulence activity of the G2A mutant by inoculating the bacterial strains into tomato PtoR plants and tobacco W38 plants. As shown in Figures 7B and 7C, *Pseudomonas* strains carrying the AvrPto mutant (G2A) induced neither disease resistance nor HR in tomato or tobacco plants. The results indicate that the putative myristoylation site is required for the avirulence function of AvrPto in *Pseudomonas*.

The *avrPto* mRNA contains the type III secretion signal within the first 15 bases of the open reading frame (Anderson et al., 1999). Thus, the G2A mutation (from GGA to GCA) may have altered protein secretion, which would account for the loss of the avirulence function of the *avrPto* gene in *Pseudomonas*. We tested this possibility by analyzing the secretion of the G2A mutant protein from the T1 strain of *P. s. tomato*. Protein gel blot analysis with the anti-AvrPto antibodies showed the presence of the wild-type AvrPto and G2A proteins in both the bacterial cells and the minimal...
plants carrying the G2A mutant transgene did not develop HR when treated with tetracycline (data not shown).

We also tested whether the G2A mutation affected the Pto–AvrPto interaction, which could provide an alternative explanation for the loss of avirulence activity (Scofield et al., 1996; Tang et al., 1996). The yeast two-hybrid assay showed that both G2A and the wild-type AvrPto proteins interacted equally with Pto (Figure 7G).

These assays on G2A protein localization, avirulence function, secretion, and Pto interaction suggest that the putative myristoylation site is required for the membrane localization of AvrPto and that the association of AvrPto with the plasma membrane is essential for resistance gene recognition and subsequent activation of disease resistance.

DISCUSSION

In this study, we have described three regions in AvrPto that play important roles in avirulence function. The central region, defined by amino acid residues from Ser94 to Gly99, is required for the specific recognition between AvrPto and the tomato Pto protein but is not required for recognition in tobacco. In contrast, the C-terminal region from Pro146 to Ser153 is indispensable for the avirulence activity in tobacco plants but is not required for tomato Pto recognition. These two regions probably are involved in the association with the corresponding R gene products in tomato and tobacco plants. The putative myristoylation site at the N terminus is essential for the plasma membrane association of AvrPto and the avirulence activity in both tobacco and tomato plants, suggesting that the recognition between AvrPto and the R gene products is associated with the plasma membrane.

Using the reverse yeast two-hybrid assay, we identified many mutations in AvrPto that disrupted the interaction with Pto. Similarly, the screening based on the HR-inducing activity in tobacco W38 and W38(35S::Pto) plants identified a large collection of mutants that exhibited no recognition by the tobacco and tomato resistance genes. These mutations are located throughout the AvrPto protein. Many mutations rendered the AvrPto protein unstable; perhaps they involved amino acid residues that are required to maintain the overall structure and stability of the AvrPto protein. In contrast, mutations at the central region and the C-terminal region of AvrPto had minor or no effect on AvrPto stability. Mutants in the central region disrupted the avirulence activity in tomato but not in tobacco. Conversely, mutants in the C-terminal region disrupted the avirulence activity in tobacco but retained avirulent in tomato. We also tested whether the differential protein stability of AvrPto mutants in tomato and tobacco plants accounted for the differential avirulence activities of the two groups of mutants. Examination of representative AvrPto mutants of each group did not reveal a differential protein stability in plants; apparently, therefore,

<table>
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<th>avrPto Mutant</th>
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<th>HR-Inducing Activity</th>
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<td></td>
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<td>E104 frameshift</td>
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*a Ten avrPto mutants that did not induce the HR in both W38 and W38(35S::Pto) were randomly selected and sequenced.

b Single-letter code is used to designate the amino acids.
the two groups of AvrPto mutations disrupt the local structures that determine gene-for-gene recognition specificity. The two small regions defined by these mutations probably represent the structures that are recognized specifically by Pto and the tobacco R gene product.

Computer analysis predicted that the central region of AvrPto specifying the Pto recognition forms a turn flanked by two α helices. Similarly, the C-terminal region required for the avirulence activity in tobacco also spans a turn that is bordered by two β sheets. Thus, these motifs probably are loops recognized by the tomato Pto protein and the tobacco R gene product, respectively. If so, they are similar to the active sites of the Avr9 peptide that are required for avirulence activity in Cf9 plants. Two solvent-exposed loops of Avr9 are essential for its binding to the plant plasma membrane and for the HR-inducing activity in Cf9 plants (Kooman-Gersmann et al., 1997, 1998; van den Hooven et al., 1999). A direct role of the AvrPto central motif in protein–protein interaction is supported by the yeast two-hybrid results. Mutations in this motif abolished the interaction with the tomato Pto protein. The central motif of AvrPto may interact with the kinase activation domain, the specificity determinant of the Pto protein (Frederick et al., 1998; Rathjen et al., 1999). An examination of whether the C terminus of AvrPto interacts with the tobacco R gene product awaits the isolation of the tobacco R gene.

The identification of the two recognition sequences in AvrPto led to speculation on the evolution of R gene specificity. Like many avr genes, avrPto is not only an avirulence factor, it also has the virulence function (Chang et al., 2000; Shan et al., 2000). We showed previously that AvrPto mutants S94P, I96T, and G99V disrupted the avirulence function but not the virulence function in tomato, suggesting that the two opposite functions are separated structurally (Shan et al., 2000). We show here that the same mutants disrupted the recognition by Pto but not by the tobacco R gene. These findings lead us to speculate that R gene evolution might not target the virulence structure of an Avr protein. Rather, R genes might have evolved to recognize a structurally compatible sequence of the Avr protein. Therefore, if an Avr protein is recognized by multiple R genes, these R genes, depending on their structure, might recognize different regions of the Avr protein. The identification of two distinct sequences of AvrPto that are differentially recognized by Pto and the tobacco R gene supports this speculation.

The R gene in tobacco W38 plants has not been cloned. Several lines of evidence suggest that the tobacco R gene product is similar to the tomato Pto kinase. The two species both belong to the Solanacea family, and tobacco carries sequences that are highly homologous with the tomato Pto gene (Martin et al., 1993). In tomato, Pto and Prf are tightly linked (Jia et al., 1997). At least some Pto- and Prf-like sequences also are linked in tobacco (X. Tang, unpublished results), suggesting a conserved gene organization in the two plant species. Furthermore, the tomato Pto gene functions correctly when introduced into tobacco plants (Rommens et

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**Figure 5.** The C-Terminal Deletion in AvrPto Specifically Abolishes Recognition by the Tobacco R Gene.

(A) Growth of P. s. tabaci strain 11528R carrying avrPto deletion mutants in tobacco W38 plants.

(B) Growth of P. s. tomato strain T1 carrying avrPto deletion mutants in tomato PtoR plants.

Inoculation and bacterial measurement were as described in Figure 4. Error bars indicate SE.
al., 1995; Thilmony et al., 1995). If the tobacco R gene is similar to Pto, a structural polymorphism between Pto and the tobacco R gene product might explain the differential recognition specificity of the two R genes.

Equally possible, the R gene in W38 could be an ortholog of Prf. Prf is highly similar to the nucleotide binding site–leucine-rich repeat (NBS-LRR) type of R genes and is indispensable for Pto-mediated resistance (Salmeron et al., 1996). The Prf protein in tomato may be part of the receptor complex that also includes Pto, and the C-terminal motif of AvrPto may help to stabilize the protein complex by interacting with the Prf protein. This is not a remote possibility. Although AvrPto mutants with amino acid substitutions or deletions at the C terminus were identical to the wild-type AvrPto in interaction with the tomato Pto in the yeast two-hybrid system, the resistance in tomato plants incited by these mutants was noticeably weaker. One plausible explanation is that the association of Prf or AvrPto with the receptor protein complex is less stable when Prf does not bind the altered C terminus of AvrPto. This explanation is reinforced by the recent report that an NBS-LRR type of R gene product binds the cognate Avr protein (Jia et al., 2000). If the tobacco resistance activity is encoded by a Prf-like protein that interacts with AvrPto, this will provide direct evidence that Pto, Prf, and AvrPto belong to the same signal receptor complex. Such a finding would support the current model of R gene pathways, which involves both NBS-LRR proteins and protein kinases (Grant and Mansfield, 1999).

The possibility also exists that the tobacco R gene may be entirely unrelated to the Pto or Prf sequences. If so, this suggests that R genes in different plant species can evolve by gene convergence rather than conservation.

Myristoylation of the bacterial avirulence proteins AvrRpm1, AvrB, and AvrPphB was demonstrated recently as a mechanism that anchors the proteins to the Arabidopsis plasma membrane (Nimchuk et al., 2000). In an independent study described here, we found that this modification probably occurs in the AvrPto protein and is critical for localizing AvrPto to the plasma membrane. Mutation of the putative myristoylation site at the N terminus of AvrPto disrupted the membrane association. Although this mutation did not affect the protein secretion in Pseudomonas bacteria, the Pto interaction in the yeast two-hybrid system, or the protein stability in plant cells, the mutant protein exhibited no

Figure 6. AvrPto Protein Is Localized in the Plasma Membrane. (A) Tetracycline-induced HR. Transgenic tobacco plants (W38) carrying the tetracycline repressor (TetR) transgene plus an empty vector (vector) or the avrPto transgene were injected with either 10 mM MgCl2 (Buffer) or 1 mg/L tetracycline (Tc). Leaves were photographed 17 hr after tetracycline treatment.

(B) Membrane localization of AvrPto. (Left) Tobacco transgenic plants containing empty vector (vec) or the avrPto transgene (avr) were injected with 10 mM MgCl2 (Tc, –) or 1 mg/L tetracycline (Tc, +). Leaf tissues were collected 10 hr after injection. Total protein was extracted as described in Methods, separated by SDS-PAGE, and analyzed by protein gel blotting with anti-AvrPto antibodies. (Right) Total protein was extracted from leaves containing the plain vector or the wild-type avrPto gene and separated by centrifugation into soluble (S) and membrane (M) fractions. Protein in the membrane fraction was resuspended in an equal volume of protein extraction buffer containing 1% Triton X-100, separated by SDS-PAGE, and analyzed by protein gel blotting with anti-AvrPto antibodies. Arrowheads indicate AvrPto.

(C) Localization of AvrPto to the plasma membrane. Plasma membrane was separated from intracellular membrane by the two-phase partitioning system. (Top) Localization of the AvrPto protein. Lanes 1 through 4: soluble fraction, plasma membrane, intracellular membrane, and total membrane, respectively. Lanes 1 and 4 were loaded with 20 μg of protein; lanes 2 and 3 received 10 μg of protein. (Bottom) Marker enzyme activities in plasma and intracellular membranes. PM and INTRA denote plasma and intracellular membranes, respectively. The activity of vanadate-sensitive ATPase is expressed as nmol phosphate·min⁻¹·mg⁻¹ protein. The activity of cytochrome (Cyt)c oxidase and NADH-dependent cytochrome c reductase is expressed as μmol cytochrome c·min⁻¹·mg⁻¹ protein.
AvrPto Specificity and Localization

The association of AvrPto with the plasma membrane is essential for R gene recognition and the subsequent activation of the disease resistance pathways. Myristoylation seems to be a common modification to bacterial avirulence proteins in plant cells. Four Pseudomonas avirulence proteins (AvrB, AvrC, AvrRpm1, and AvrPto) contain a putative myristoylation motif at their N termini (Nimchuk et al., 2000). Three of these proteins (AvrPto, AvrB, and AvrRpm1) require the myristoylation motif for the avirulence function. The functional relevance of the putative myristoylation motif in AvrC has not been tested. The myristoylation modification has been demonstrated in plant cells for AvrRpm1 and AvrB (Nimchuk et al., 2000). Although myristoylation of AvrPto has not been determined in planta, given the structural and functional similarities of the myristoylation site among these Avr proteins, it will not be surprising if AvrPto is also myristoylated in planta.

It is interesting that Pto also contains a myristoylation site at its N terminus, which has been suggested to play a role in targeting the kinase to a plant membrane (Martin et al., 1993). Previous experiments in which a Pto(G2A) mutant was expressed from the 35S promoter indicated that the motif is not required for AvrPto-mediated disease resistance.

Figure 7. The G2A Mutation Disrupts Membrane Localization and the Avirulence Function of AvrPto.

(A) The G2A mutation disrupts the membrane association of AvrPto. Tobacco transgenic plants containing the avrPto gene or the G2A mutant were inoculated with 1 mg/L tetracycline. Leaf tissues were collected 10 hr after injection. Membrane protein was separated from soluble protein by centrifugation and examined by using anti-AvrPto antibodies. S, soluble protein; M, membrane protein.

(B) The myristoylation mutation disrupts the avirulence activity in tomato. T1 strains carrying no avrPto (−avrPto), the wild-type avrPto (avrPto), or the mutant avrPto (G2A) were vacuum-infiltrated into tomato PtoR plants at 10^8 cfu/mL. Disease symptoms were documented 4 days after inoculation.

(C) The G2A mutation disrupts the avirulence activity in tobacco. P. s. tabaci 11528R strains carrying no avrPto (−avrPto), the wild-type avrPto (avrPto), or the mutant avrPto (G2A) were inoculated into W38 plants at a concentration of 10^6 cfu/mL. The HR was photographed 18 hr after inoculation.

(D) The G2A mutation does not affect the secretion of AvrPto. P. s. tomato T1 strains carrying no avrPto (−avrPto), the wild-type avrPto (avrPto), or the mutant avrPto (G2A) were grown in minimal medium. Bacterial cells (B) and supernatant (S) were examined for the presence of the AvrPto protein by using protein gel blot analysis.

(E) The secretion of the G2A protein is Hrp-dependent. ΔavrPto/G2A designates the DC3000ΔavrPto strain containing the G2A mutant; hrcC/G2A designates the hrcC mutant strain of DC3000 containing the G2A mutant; hrcC designates the hrcC mutant strain of DC3000. Secretion of AvrPto and the G2A protein were measured as described in (D).

(F) Direct expression of the G2A mutant protein in plant cells does not cause the HR. The wild-type avrPto and mutants G2A and 1603 (Table 2) were cloned into a plant expression vector and introduced into the Agrobacterium EHA105 strain. The resulting strains were injected into W38 plants. The HR was documented 3 days after injection.

(G) The G2A mutation does not affect the Pto interaction. The G2A mutant was cloned into the prey vector pJG4-5, and the interaction with Pto was assayed with the yeast two-hybrid system. A noninteracting member of the Pto family (pto; Jia et al., 1997) was included as a control.
(Loh et al., 1998). Although the Pto protein was not detectable by antibodies in these experiments, as Loh et al. noted, overexpression of the protein may have obscured an effect of the myristoylation site. Experiments are underway to further investigate the role of the Pto G2 residue by expression of a Pto(G2A) mutant gene by using the native Pto promoter (G.B. Martin, unpublished data). If the Pto myristoylation motif is not required for disease resistance, then a possible model supported by our present results is that membrane-associated AvrPto recruits Pto to a receptor complex, where it activates the resistance signaling pathway.

**METHODS**

**Generation and Screening of AvrPto Mutants Based on Pto Interaction**

The prey plasmid (pJG4-5) containing the wild-type avrPto gene (Tang et al., 1996) was used as a template to generate an avrPto mutant library by polymerase chain reaction (PCR). The primers used were 5'-AGAATTCTGCGGAAATATATGCGCC-3' (EcoRI site underlined) and 5'-ACTCGAGTGCGACATTTGACGCC-3' (XhoI site underlined). The PCR was assembled in 1× PCR buffer (Promega), 10 ng of template plasmids, 4 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 0.5 μM primers, and 2.5 units of Taq DNA polymerase (Pwo; Boehringer Mannheim) and primers 5'-AGAATTCTGCGGAAATATATGCGCC-3' (EcoRI site underlined) and 5'-ACTCGAGTGCGACATTTGACGCC-3' (XhoI site underlined). The PCR products were digested with EcoRI and XhoI, and subcloned into the pJG4-5 plasmid (Golemis et al., 1997). Approximately 100,000 clones were obtained after transformation into Escherichia coli. The clones were pooled before plasmid purification. The pooled plasmid DNA (containing mutagenized avrPto as well as the wild-type avrPto in pJG4-5) was transformed en masse into the yeast strain carrying Pto in the bait plasmid pEG202 and the LacZ reporter gene (Golemis et al., 1997). The procedures described by Lundblad (1997) were followed for yeast transformation. Approximately 4000 yeast colonies were collected and tested individually on X-Gal plates. Prey plasmids containing avrPto mutants were isolated from the white or light-blue colonies according to Lundblad (1997) and transformed into the yeast strain containing the Pto bait for confirmation. The confirmed avrPto mutants were sequenced.

The amount of AvrPto protein in yeast was determined by protein gel blot analysis. Yeast cells growing in the inducing medium (Golemis et al., 1997) were centrifuged, and total protein was extracted by boiling the yeast pellet in 1× protein sample buffer (Sambrook et al., 1989). The total protein was separated by SDS-PAGE, transferred to a poly(vinylidene difluoride) membrane, and hybridized with affinity-purified anti-AvrPto antibodies (Shan et al., 2000). The membrane was hybridized with the secondary antibodies and developed with the enhanced chemiluminescence reagent according to the manufacturer’s instruction (Amersham).

**Hypersensitive Response–Based Mutant Screening in Tobacco**

The pooled pJG4-5 plasmids containing the avrPto mutants were digested with EcoRI and XhoI, and the insert was subcloned into the pGem7Z plasmid (Promega), resulting in a library of 100,000 clones. pGem7Z plasmids containing the avrPto mutant library were isolated from the pooled clones and digested with BamHI and XbaI. The inserts were purified and introduced into the pPtE6 plasmid (the broad-host-range pDSK519 plasmid containing the wild-type avrPto; Ronald et al., 1992) by replacing the wild-type avrPto. Approximately 4000 clones (avrPto mutant library in pPtE6 plasmid) were obtained after transformation into E. coli. These clones were pooled for plasmid isolation. The plasmid mixture (pPtE6 carrying avrPto mutants) was introduced into Pseudomonas syringae pv tabaci strain 11528R by electroporation. The transformed P. s. tabaci colonies were selected in King's B (KB) medium containing 100 mg/L rifampicin and 25 mg/L kanamycin. The resulting P. s. tabaci colonies were resuspended individually in 1 mL of sterile water and injected into fully expanded leaves of 10-week-old tobacco (Nicotiana tabacum) W38 plants and transgenic W38 plants containing 35S::Pto (Thilmont et al., 1995). Colonies that failed to induce a hypersensitive response (HR) on W38 plants but induced an HR on W38(35S::Pto) were reconfirmed by using an inoculum of 10⁶ colony-forming units (cfu)/mL. pPte6 plasmids containing avrPto mutants were isolated from the confirmed P. s. tabaci colonies, the insert DNA containing avrPto mutants was amplified by PCR using primers 5'-AGAATTCTGCGGAAATATATGCGCC-3' and 5'-ACTCGAGTGCGACATTTGACGCC-3', and the PCR product was sequenced.

To test the interaction of the isolated AvrPto mutants with Pto, we amplified each avrPto mutant by PCR with a high-fidelity DNA polymerase (Pwo; Boehringer Mannheim) and primers 5'-AGAATTCTGCGGAAATATATGCGCC-3' (EcoRI site underlined) and 5'-ACTCGAGTGCGACATTTGACGCC-3' (XhoI site underlined). The PCR products were digested with EcoRI and XhoI and introduced into the prey plasmid pJG4-5. The resulting clones were verified by sequencing analysis before transformation into yeast containing the Pto bait.

**Introduction of avrPto Mutants into P. s. tomato and P. s. tabaci**

pJG4-5 plasmids containing the avrPto mutants identified with the reverse yeast two-hybrid screen were digested with EcoRI and XhoI, and the inserts were subcloned into the pGemTZ plasmid (Promega). The resulting constructs were digested with BamHI and XbaI, and the inserts containing avrPto mutants were introduced into pPte6 plasmid (Ronald et al., 1992), replacing the wild-type avrPto gene. The resulting constructs were introduced into P. s. tomato strain T1 and P. s. tabaci strain 11528R by triparental mating (Willis et al., 1988). The transformed Pseudomonas colonies were selected in KB medium containing 100 mg/L rifampicin and 25 mg/L kanamycin. The avrPto mutants in Pseudomonas strains were confirmed by PCR and sequencing analysis.

**Bacterial Inoculation and Measurement**

The culture of P. s. tabaci strains and the preparation of inoculum were as described (Thilmont et al., 1995). The bacterial suspension was inoculated into tobacco leaves with a needleless syringe. Inoculum of 10⁶ cfu/mL was used for the HR assay. Inoculum of 10⁶ cfu/mL was used for the disease symptom assay and for the measurement of bacterial growth in tobacco leaves. To measure the growth of P. s. tabaci strains in tobacco leaves, we collected six leaf discs (1 cm²) from three inoculated plants. Two discs were ground in 1 mL of sterile water, diluted to the desired concentration, and plated on KB medium containing appropriate antibiotics. To measure the bacterial
growth in tomato (Lycopersicon esculentum), we inoculated 6-week-old tomato plants with P. s. tomato strains \(10^4\) cfu/mL by vacuum infiltration. Bacterial culture, preparation of inoculum, and measurement of bacteria numbers in tomatoes were as described (Tang et al., 1999).

**Protein Secretion Assay in Pseudomonas**

Pseudomonas bacteria were grown for 1 day in KB medium containing appropriate antibiotics. The bacteria were harvested by centrifugation and washed twice with the minimal medium containing 10 mM fructose, pH 6.0 (Huynh et al., 1989). The bacteria were resuspended and diluted in the same medium to OD\(_{600}\) = 0.2 and grown at room temperature for 16 hr. To measure the secretion of AvrPto mutant proteins, we first adjusted bacterial cultures to the same concentration in the minimal medium. Equal volumes of the adjusted bacterial cultures were centrifuged at 2000g (to avoid bacterial leakage) for 5 min. The supernatant was transferred to a clean microcentrifuge tube and spun at 16,000g for 5 min to remove the residual bacteria. The bacterial pellet was resuspended in an equal volume of distilled water. Sixty microliters of the liquid medium or bacterial suspension was mixed with 30 \(\mu\)L of 3 \times protein sample buffer (Sambrook et al., 1989) and boiled for 5 min, and the protein was separated by SDS-PAGE. The presence of AvrPto mutant proteins was examined by using anti-AvrPto antibodies.

**Tetracycline-Induced Expression of AvrPto and G2A Proteins in Tobacco**

Transgenic tobacco W38 seed carrying the bacterial tetracycline repressor (TetR) under the control of the 3SS promoter was kindly provided by Dr. Christiane Gatz. Transgenic plants containing a single insertion but expressing large amounts of TetR were identified by DNA gel blot and RNA gel blot analysis with TetR probes. avrPto or G2A cDNA was cloned into Bin-Hyg-TX plasmid under the control of the tetracycline-inducible promoter (Gatz et al., 1992) and transformed into W38 plants containing the TetR gene. The plain Bin-Hyg-TX plasmid also was transformed into the TetR plants as a control. Transgenic plants were selected by using kanamycin and hygromycin. Expression of AvrPto or the G2A protein was induced by infiltrating tetracycline (1 mg/L) into the leaves of the transgenic plants.

**Protein Fractionation Analysis**

To measure the AvrPto and G2A proteins in plants, we inoculated the leaves of the transgenic plants with 1 mg/L tetracycline and harvested them 10 hr after treatment. The leaf tissue was frozen and powdered with liquid nitrogen. Total protein was extracted with 1\% Protease Inhibitor Mix for Plant (Sigma). The plant debris were removed by centrifugation at 10,000g at 4°C. The membrane fraction was separated from the soluble proteins by centrifugation at 100,000g at 4°C for 1 hr. The soluble fraction was transferred to a clean tube, and the membrane fraction was resuspended in an equal volume of the above protein extraction buffer containing 1\% Triton X-100. Sixty microliters of soluble fraction or membrane fraction was mixed with 30 \(\mu\)L of 3 \times protein sample buffer (Sambrook et al., 1989) and boiled for 5 min, after which the proteins were separated by SDS-PAGE. The presence of AvrPto and the G2A mutant protein was examined by protein gel blot analysis with anti-AvrPto antibodies (Shan et al., 2000).

A two-phase partitioning method (Larsson et al., 1987) was used to separate the plasma membrane from the intracellular membrane. The procedures described by Xu et al. (1996) were followed for membrane preparation and fractionation. The assays for the marker enzymes vanadate-sensitive ATPase, NADH-dependent cytochrome c reductase, and cytochrome c oxidase were performed according to reported procedures (Briskin et al., 1987; Xu et al., 1996).

**Site-Directed Mutagenesis of avrPto**

To introduce the myristoylation mutant G2A into Pseudomonas bacteria, we digested pPic6 plasmid (Ronald et al., 1992) with EcoRI and Xbali, and the insert was cloned into pGem7Z plasmid (Promega). The resulting construct was used as the template for site-directed mutagenesis. The QuickChange site-directed mutagenesis kit (Stratagene) and two complementary primers carrying the point mutation (indicated in boldface), 5′-GGGGTATACGAAATTCGATGTCGGGCGATCC-3′ and 5′-GGATCGCCGACACATATTGTCCATTGTATACCTC-3′, were used to generate the myristoylation mutation in avrPto according to the manufacturer’s instructions. Mutant avrPto in pGem7Z plasmid was confirmed by sequence analysis. The confirmed plasmid was digested with EcoRI and Xbali, and the insert was constructed in the pPic6 plasmid by replacing the wild-type avrPto. The resulting construct was introduced into P. s. tomato and P. s. tabaci by triparental mating (Willis et al., 1988).

To clone the myristoylation mutant into prey plasmid pJG4-5, we used avrPto in pJG4-5 as a template in a PCR with 5′-AGGAATTCTCATGGCAAATATATGTGTCGGGCGAT-3′ (EcoRI site underlined, myristoylation mutation site in boldface) and 5′-AATCATGCGCTGCAATTATGGAAGATCG-3′ (XhoI site underlined) primers. The PCR product was digested with EcoRI and XhoI and cloned into the pJG4-5 plasmid (Golemis et al., 1997). The resulting construct was confirmed by sequence analysis before transformation into the yeast strain containing the Pto bait.

**Agrobacterium-Mediated Transient Gene Expression in Plant**

avrPto, the G2A mutant, and the avrPto mutant 1603 (Table 2) were cloned in plasmid pBI121 and introduced into the Agrobacterium strain EHA105 as described (Tang et al., 1996). Transient expression of the AvrPto and mutant proteins in tobacco W38 plants was as described (Tang et al., 1996).

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