RESEARCH ARTICLE

Use of a Gene Expression System Based on Potato Virus X to Rapidly Identify and Characterize a Tomato Pto Homolog That Controls Fenthion Sensitivity

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A novel transient gene expression system was used to study both the tomato disease resistance gene Pto and a Pto homolog designated Fen. The gene expression system was based on potato virus X (PVX). Tomato plants that were both susceptible to strains of Pseudomonas syringae pv tomato carrying the corresponding avirulence gene avrPto and insensitive to the insecticide fenthion were infected with in vitro-generated transcripts of PVX derivatives containing either Pto or Fen. Expression of the Pto gene from the virus genome failed to elicit R. s. tomato resistance, indicating that the PVX system is not suitable for the study of Pto. However, expression of the Fen gene resulted in sensitivity to fenthion. The utility of the PVX gene expression system was further demonstrated through structure/function studies of the Fen gene. A correlation was shown between Fen protein kinase activity and the ability of this protein to confer fenthion sensitivity to tomato. Furthermore, it was demonstrated that mutation of a putative N-terminal myristoylation site, proposed to be involved in membrane targeting, rendered the Fen protein inactive. Analysis of a Pto-Fen chimeric gene allowed the fenthion sensitivity domain to be localized to the C-terminal part of the Fen protein. Interestingly, expression of the Fen kinase from the PVX genome in Nicotiana spp resulted in a fenthion-independent necrotic response. Our results support the involvement of the Fen gene in a signal transduction pathway(s).

INTRODUCTION

Pseudomonas syringae pv tomato is the causal agent of bacterial speck disease of tomato and results in significant yield losses, especially in cool and moist regions (Goode and Sasser, 1980). The disease resistance gene Pto that confers resistance to P. s. tomato strains has been introgressed into breeding lines from the wild tomato species Lycopersicon pimpinellifolium (Pitblado et al., 1984). The Pto locus maps to chromosome 5 and was found to be incompletely dominant for resistance to P. s. tomato (Pitblado et al., 1984; Carland and Staskawicz, 1993). Expression of Pto resistance is correlated with the production of a hypersensitive response (HR), which is observed as a rapid and localized necrosis of host tissue. A gene-for-gene relationship determines the incompatible interaction between P. s. tomato and tomato, implying molecular recognition between the signal delivered by the avirulence gene avrPto in the pathogen and the corresponding Pto resistance gene in the host (Ronald et al., 1992).

Recently, loci from both the host and the pathogen have been cloned and characterized. The avrPto gene encodes a predicted translation product that is a mostly hydrophilic, 18.3-kD protein with no similarities to known protein sequences (Ronald et al., 1992; Salmeron and Staskawicz, 1993). Cloning of the Pto locus was accomplished using a positional cloning strategy (Martin et al., 1993a). The Pto locus consists of a cluster of five to seven homologous genes. One cDNA that maps at this locus, when transformed into a normally susceptible tomato line, conferred resistance to P. s. tomato carrying avrPto and was designated Pto. The predicted translation product of Pto has high levels of homology to serine/threonine protein kinases, suggesting a role in signal transduction (Martin et al., 1993a).

A second phenotype cosegregates with Pto resistance. Plants containing the Pto locus, which exhibit resistance to P. s. tomato expressing avrPto, are sensitive to the organophosphorous insecticide fenothion (Laterrot and Philouze, 1985; Carland and Staskawicz, 1993; Martin et al., 1993b). Application of fenothion leads to a necrotic response that is macroscopically similar to the HR elicited by P. s. tomato carrying avrPto (Carland and Staskawicz, 1993). Genetic studies demonstrated that P. s. tomato resistance and fenothion sensitivity were separated by less than 0.2 centimorgans, suggesting that the two traits were encoded by the same gene or two tightly linked genes (Carland and Staskawicz, 1993;
To study Pto and other members of the Pto gene family, the gene controlling fenthion sensitivity (Fen) is in fact distinct from the Pto gene. Five independent Pto mutants, identified by screening a large population of M₂ families, retain full sensitivity to fenthion (Salmeron et al., 1994). Therefore, Pto and Fen, although in close physical proximity, appear to be encoded by two different genes. Recently, Martin et al. (1994) showed by stable transformation that the Fen gene is one of the Pto homologs.

A third component involved in both Pto resistance and fenthion sensitivity has been identified and genetically characterized by the study of the same M₂ population described previously (Salmeron et al., 1994). Mutant plants were shown to carry mutations in a novel locus designated Prf (Pseudomonas resistance and fenthion sensitivity). They were simultaneously altered in Pto resistance and fenthion sensitivity, suggesting that Prf plays a role in the signal transduction pathways involving Pto and Fen.

We have employed a recently developed transient gene expression system based on a plant viral vector to study Pto and other members of the Pto gene family. Episomal plant viral vectors provide an alternative to stable transformation of foreign genes in plants and offer the advantages of speed and facility (Ahlquist and Pacha, 1990). A plant viral vector was used that was produced from potato virus X (PVX; Chapman et al., 1992). PVX is a single-stranded RNA virus and belongs to the potex virus group (Koenig and Leseman, 1989). The PVX vector contains a duplicated coat protein promoter that directs high-level expression of foreign genes. Successful applications of this vector include expression of the Escherichia coli β-glucuronidase (Gus) gene in Nicotiana clevelandii (Chapman et al., 1992) and of the fungal avirulence gene avr9 in tomato (Hammond-Kosack et al., 1995). Although the PVX system did not prove useful for analysis of the Pto gene, it allowed us to identify and characterize Fen, a member of the Pto gene family that confers fenthion sensitivity in tomato. Interestingly, PVX-directed expression of Fen in various fenthion-insensitive Nicotiana spp resulted in a fenthion-independent necrotic response.

RESULTS

Pto Open Reading Frame 1 Encodes an Active Protein Kinase That Is Functional in Conferring Disease Resistance

To study Pto and other members of the Pto gene family, the Pto gene was cloned using the polymerase chain reaction (PCR). Primers specific to the 5' and 3' ends of the open reading frame 1 (ORF1) from Pto (Martin et al., 1993a) were used to generate a PCR product from DNA of the resistant tomato line Rio Grande 76R. The PCR product was shown by sequencing to be identical to ORF1 (GenBank accession number U02271) and called Pto-ORF1.

To determine whether the Pto-ORF1 product has kinase activity and can autophosphorylate, Pto-ORF1 was cloned into a glutathione S-transferase (Gst) vector producing a fusion protein of 62 kD. Pto contains a Lys residue at position 69 in kinase domain II that is highly conserved among protein kinases and is crucial for catalytic function (Taylor et al., 1992). A mutated Pto-ORF1 kinase was constructed by substituting an Asn residue for Lys-69 and was cloned into the Gst vector. Soluble fusion proteins were extracted from E. coli carrying the Gst fusions by incubation with glutathione–agarose beads. In vitro kinase activity was tested by adding γ-32P-ATP, separating proteins by SDS–PAGE, and subjecting the gel to autoradiography. We found that the wild-type Pto-ORF1 fusion protein can autophosphorylate (Figure 1), whereas the Pto-ORF1 kinase–mutated fusion protein did not autophosphorylate. The lack of kinase activity of the Pto-ORF1 kinase mutant indicates that phosphorylation of fusion proteins was not the result of bacterial kinase activity. Thus, the Pto-ORF1 gene encodes an active kinase.

To test whether Pto-ORF1 was functional, it was cloned into the binary vector SL44024 (Jones et al., 1992) under the transcriptional control of the 35S promoter of cauliflower mosaic virus. The susceptible line Rio Grande 76S (pto/pto) was transformed by a standard Agrobacterium-mediated procedure. To test for complementation to resistance, leaves of two independent transformants were inoculated with R. s. tomoato race 1 strain T1 carrying the avrulence gene avrPto and were assayed for macroscopic resistant or susceptible phenotypes after 4 days. No disease symptoms developed in these transformants (data not shown), verifying that Pto-ORF1 is functional in conferring resistance to T1(avrPto).

Figure 1. Analysis of Pth1 and Pto Kinase Activity.

 Autoradiogram of an SDS-polyacrylamide gel. The lanes contain equal amounts of Gst fusion proteins extracted with glutathione–agarose beads that were tested for autophosphorylation activity by the addition of γ-32P-ATP. Fusion proteins contain the Fen kinase mutant (lanes 1 and 2), the Fen wild type (lanes 3 and 4), the Pto kinase mutant (lanes 5 and 6), and the Pto wild type (lanes 7 and 8). Lanes 9 and 10 contain Gst purified from E. coli. Lane 11 is a control lane with the extraction performed on an E. coli DH5α culture expressing neither Gst nor a Gst fusion protein. Cultures had been (+) or had not been (−) induced with isopropyl β-d-thiogalactopyranoside (IPTG). The 62 kD shows the position expected for migration of a 62-kD protein based on electrophoresis of Bio-Rad protein standards.
Characterization of a Pto Homolog Using PVX

Cloning of Pto Homologous Genes

To isolate other members of the Pto gene family, 1.2 x 10^6 plaque-forming units (pfu) from a cDNA library made from leaves of the resistant line 76R treated with T1(avrPto) were screened with a genomic DNA fragment homologous to Pto (J.M. Salmeron, C.M.T. Rommens, and B.J. Staskawicz, unpublished results). Ten hybridizing cDNAs were identified, eight of which contained identical sequences and were designated Pth1 (Pto homolog 1). Two other expressed Pto homologs, Pth2 and Pth3, were isolated and will be described elsewhere.

The Pth1 gene was sequenced and a 954-bp ORF was found. Pth1 encodes a predicted protein of 318 amino acids that displays 80% amino acid identity to the Pto protein (Figure 2).

Stability of PVX Derivatives in Tomato

To facilitate functional analyses of Pto and Pth1, the corresponding genes were cloned into the PVX vector behind the duplicated PVX coat protein promoter. Prior to analysis of these genes, however, we examined the stability of a PVX derivative carrying the Gus gene in tomato. Leaves of the susceptible line 76S were infected with a PVX derivative containing the Gus gene, PVX:Gus (Chapman et al., 1992). Three weeks after infection, Gus activity in individual leaflets displaying virus-induced mosaic symptoms was analyzed using a histochemical stain. High levels of staining were found in leaves of six of seven systemically infected plants (Figure 3). PVX derivatives appeared to spread throughout 76S plants and allowed expression of the foreign gene in large areas of the leaves. However, in contrast to the uniform distribution of the virus-induced mosaic across the leaf surface, Gus staining was not uniform, suggesting that there may be loss of the functional Gus gene in part of the viral population.

Figure 2. Comparison of Deduced Amino Acid Sequences of Pth1 to That of Pto.

As defined by Hanks et al. (1988), the kinase sequences have been divided into 11 domains, and the amino acids that are conserved in protein kinases (#) are shown in the top line. Underlining represents amino acids that are absolutely conserved, unitalicized type represents conserved amino acids, and lowercase letters refer to amino acids found in serine/threonine kinases. The consensus sequence for myristoylation sites is indicated in italics. The most variable region between Pto and Fen is indicated with asterisks at the beginning and end of the region. Amino acids of the Fen protein that are not identical to Ro have been indicated by letters; identical amino acids are indicated by dashes. Dots refer to gaps.

Figure 3. Gus Activity in Leaves of Tomato Plants Infected with PVX:Gus.

Shown are systemically infected leaves of seven different tomato plants stained for Gus activity.
Functional Analysis of the \( Pto \) Gene in Virus-Infected Plants

To functionally assay the \( Pto \) gene, cotyledons of 2-week-old 76S tomato seedlings that were susceptible to \( T1(\text{avr}Pto) \) and insensitive to fenthion were infected with the PVX derivative PVX: \( Pto \). The stability of the \( Pto-\text{ORF}1 \) insert in systemically infected plants was tested by performing PCR on reverse-transcribed RNA (RT-PCR) from systemically infected leaflets. Products with sizes predicted for full-length inserts could be amplified from reverse-transcribed RNA isolated from eight of 12 plants containing PVX: \( Pto \), implying that at least part of the viral population in these plants contained an intact insert (Figure 4).

We assessed the applicability of the transient PVX gene expression system for studies of the \( Pto \) gene. First, it was important to determine whether PVX infection would influence the plant response induced by \( T1(\text{avr}Pto) \) treatment. Plants systemically infected with PVX were inoculated by dipping into a suspension of \( T1(\text{avr}Pto) \), and their response was assessed after 4 days. No differences were observed in the severity of disease symptoms that developed in uninfected controls and PVX-infected plants. By inoculating leaf areas with \( 10^8 \) cfu/mL of \( T1(\text{avr}Pto) \), we also found that the resistance response (HR) of PVX-infected 76R plants was indistinguishable from that of uninfected 76R controls.

To study the effect of transient PVX-directed expression of \( Pto \) on resistance in tomato line 76S, leaves of eight 76S plants infected with PVX: \( Pto \) that had been shown to contain the intact \( Pto \) insert were inoculated with \( T1(\text{avr}Pto) \). For comparison, leaves of 76S plants infected with PVX:Gus were inoculated as well. There was no difference in disease development in 76S plants infected with PVX: \( Pto \) and in plants infected with PVX:Gus (data not shown). Because the \( Pto \) construct had been shown to be functional by transformation into tomato, this result indicates that the PVX system was ineffective for functional studies of the disease resistance gene \( Pto \) under the conditions used.

\( Pth1 \) Confers Fenthion Sensitivity

Cosegregation of \( P. s. \) \textit{tomato} resistance and fenthion sensitivity in tomato suggested to us that one of the \( Pto \) homologs might be the \( Fen \) gene. To address whether \( Pth1 \) confers fenthion sensitivity to tomato, we cloned this \( Pto \) homolog into the PVX vector and performed functional assays. Before testing the activity of \( Pth1 \), we wished to confirm that infection of tomato lines 76R and 76S with wild-type PVX would not alter fenthion phenotypes. The 76R plants systemically infected with PVX developed a mild mosaic and remained sensitive to fenthion as did the uninfected controls (Figure 5A). The 76S plants systemically infected with PVX responded to fenthion in the same manner as uninfected 76S controls and remained insensitive (Figure 5B).

Cotyledons of 2-week-old 76S tomato seedlings that were insensitive to fenthion were infected with the PVX derivative PVX: \( Pth1 \). The stability of the PVX: \( Pth1 \) insert in systemically infected plants was tested by performing RT-PCR. Products of 1.0 kb, indicating full-length inserts, were amplified from RNA isolated from 10 of 12 plants infected with PVX: \( Pth1 \) (Figure 6). Fenthion sensitivity was observed in these 10 76S plants (Figure 5C), indicating that \( Pth1 \) confers fenthion sensitivity in tomato. The number and size of the fenthion-induced necrotic lesions on leaves of PVX: \( Pth1 \) plants were similar to those of 76R plants, although lesions were sometimes less numerous in the youngest leaves of the PVX: \( Pth1 \) plants. Infection of 76R plants with PVX: \( Pth1 \) consistently resulted in an increase in the number of lesions (Figure 5D), suggesting a dosage effect for fenthion sensitivity. As expected, fenthion sensitivity did not result from infection of 76S plants with PVX:Gus or PVX: \( Pto \), and significantly, it was not observed in the two PVX: \( Pth1 \) plants that did not have recoverable \( Pth1 \) gene inserts (data not shown). Based on these results, we concluded that \( Pth1 \) is the \( Fen \) gene.

Characterization of the \( Fen \) Gene Product

The predicted amino acid sequence of the \( Fen \) protein suggests the presence of catalytic domains found in kinases. To determine if \( Fen \) has kinase activity and can autophosphorylate, \( Fen \) was synthesized as a fusion protein with Gst in \( E. coli \), and the resulting 62-kD fusion protein was assayed for kinase activity. A mutated \( Fen \) kinase was also constructed by substituting a Thr residue for Asn-167 in kinase domain VI (Figure 2). An Asn residue in kinase domain VI is highly conserved among protein kinases and is crucial for catalytic function (Taylor et al., 1992). The mutant \( Fen \) kinase gene was cloned into a Gst vector, producing a fusion protein of 62 kD. Soluble fusion proteins were extracted with glutathione—
Characterization of a Pto Homolog Using PVX

Systemically infected leaves of tomato plants were dipped into a solution containing fenthion and are shown in (A) to (G).

(A) Leaf from a 76R plant infected with PVX.
(B) and (C) Leaves from 76S plants infected with PVX and PVX:Pth1, respectively.
(D) Leaf from a 76R plant infected with PVX:Pth1.
(E) to (G) Leaves from 76S plants infected with PVX:Pth1[167T], PVX:Pth1[G2R], and the PVX:Pto/Pth1 chimeric gene, respectively.
(H) Leaf from a tobacco (N. tabacum) plant infected with PVX:Pth1.

Figure 5. Phenotypes of Plants Infected with PVX or PVX Insertion Mutants.

 agarose beads and tested for in vitro kinase activity by the addition of γ-32P-ATP. An autoradiogram of proteins separated by SDS–PAGE shows that the wild-type Fen fusion protein can autophosphorylate (Figure 1). The Fen kinase mutated fusion protein does not autophosphorylate. Thus, the Fen gene encodes a kinase that is active in vitro.

The demonstration that the Fen gene was transiently expressed in the PVX viral system has now allowed us to test for a causal relationship between the kinase activity of Fen and fenthion sensitivity in virus-infected plants. The 76S plants were infected with PVX containing the Fen kinase mutant (PVX:Pth1[167T]), and after 3 weeks, RT-PCR was performed to identify systemically infected plants with the intact PVX derivative (data not shown). Four plants with intact inserts were then assayed for fenthion sensitivity. The plants were free of necrotic lesions, indicating that the kinase activity of the Fen gene is necessary for production of symptoms of fenthion sensitivity (Figure 5E).

To test the functional significance of the putative myristoylation site of the Fen protein, a Fen mutant was constructed

Figure 6. RT-PCR Analysis of RNA from Plants Infected with PVX:Pth1.
Reactions were electrophoresed on a 1.2% agarose gel, blotted, and hybridized to Pto. Lanes 1 to 12 contain reaction products using template RNA from plants infected with PVX:Pth1; lane 13 contains the reaction product of RNA from an uninfected plant; lane 14 contains the PCR product using plasmid DNA of PVX:Pth1 as a template. The 1.1 kb indicates molecular length in kilobases based on the positions of DNA markers (Gibco BRL).
that alters the invariant Gly-2 residue to Arg. This Gly residue has been suggested to be involved in covalent linkage of myristate (Resh, 1994). The Fen mutant gene was cloned into PVX, and four 76S plants were infected with the resulting PVX derivative, PVX:Pth1(G2R). Three weeks after infection, the presence of the intact construct was confirmed by RT-PCR, and plants were treated with fenthion (data not shown). No fenthion sensitivity symptoms were observed (Figure 5F), indicating that the putative myristoylation site is crucial for the function of the Fen gene.

To further localize the region in Fen that determines specificity for fenthion, a PtolFen chimeric gene was constructed that encodes the N-terminal half of Pto (132 amino acids) and the C-terminal half of Fen (186 amino acids). Four 76S plants infected with a PVX derivative containing the chimeric gene were shown to be fully sensitive to fenthion. This indicates that the chimeric protein is functional and that the C-terminal half contains the only uniquely required region to confer fenthion sensitivity (Figure 5G).

Expression of the Fen Gene in Nicotiana spp

Nicotiana spp, such as N. tabacum and N. clevelandii, can be systemically infected with PVX. Both these plant species are insensitive to fenthion (C.M.T. Rommens and B.J. Staskawicz, unpublished results). To determine whether expression of the Fen gene leads to fenthion sensitivity of tobacco, we infected N. tabacum with PVX:Pth1. Two weeks after infection, necrotic spots developed on primary infected leaves (Figure 5H). This localized necrotic response was fenthion independent; it could not be accelerated or intensified by applying fenthion to the infected plant. One week later, mosaic symptoms were observed on younger leaves. However, these systemically infected leaves did not develop necrotic spots. By performing RT-PCR, the presence of the Fen gene in PVX could be demonstrated only in the primary infected leaves. Thus, a correlation was observed between the presence of the Fen gene in PVX and the ability of the PVX derivative to induce a necrotic response.

N. clevelandii responded even more dramatically to infection with PVX:Pth1. Ten days after infection, a necrotic reaction developed in primary infected leaves. The necrotic regions consequently expanded, and the plants died within 3 weeks (Figure 7).

No necrosis was observed in either N. tabacum or N. clevelandii infected with PVX, PVX:Gus, PVX:Pth1(N167T), or PVX:Pth1(G2R) (Figure 7; data not shown). These results suggest a three-way correlation of the kinase activity of Fen, the ability of Fen to confer fenthion sensitivity to tomato, and the ability of Fen to induce a necrotic response in Nicotiana spp.

DISCUSSION

The PVX system described in this article has facilitated the characterization of a homolog of the tomato disease resistance gene Pto. The Pto homolog, designated Fen, was shown to control fenthion sensitivity. Infection of 76S plants with a PVX derivative containing Fen resulted in fenthion sensitivity. We observed a 100% correlation between the ability to amplify an insert by RT-PCR and a fenthion-sensitive phenotype. The predicted Fen protein is 80% identical at the amino acid level to that encoded by the Pto disease resistance gene. The sequence variation between Pto and Fen is distributed in a nonrandom fashion, with the greatest variability found to reside in a stretch of amino acids between the kinase catalytic domains IX and X (Figure 2). A domain swap mutant with the N-terminal 132 amino acids of Pto and the C-terminal 186 amino acids of Fen conferred full fenthion sensitivity, suggesting that the variable region between domains IX and X may be involved in determining the specific function of Fen. We are continuing to dissect the function of this variable region using the PVX system.

Previously, comparison of the predicted Pto protein with protein sequences contained in data bases revealed similarities with the catalytic domains of many protein kinases, suggesting that the Pto protein might be a protein kinase and involved in signal transduction (Martin et al., 1993a). Here we demonstrate that both the Pto and the Fen proteins are functional kinases and are able to autophosphorylate in vitro. Kinases function in a myriad of signal transduction pathways, and based on their kinase activity, Pto and Fen are most likely components of a signal transduction pathway(s). Pto and Fen are small protein kinases and contain little more than the conserved
catalytic core that has an average size of 260 amino acids (Taylor et al., 1992). Interestingly, these proteins are 40% identical (70% homologous) to the catalytic core of the S locus receptor kinase SRK29 of *Brassica oleracea* (GenBank accession number Z30211) that is involved in self-recognition in plants. Fen contains a putative myristoylation site, suggesting that it may function in association with other proteins in the plasmalemma. One candidate for the protein interacting with Fen and Pto is Prf, a third identified component in the Fen- and Pto-controlled signal transduction pathway(s) (Salmeron et al., 1994).

Despite the similarity between the sequences of the Pto and Fen genes, we have shown that the Pto gene does not perform the functions necessary for fenthion sensitivity (Salmeron et al., 1994; this study). Similar observations have been made for the mitogen-activated protein kinase (MAPK) pathways, where no cross-talk seems to occur between homologous protein kinases active in different pathways (Blumer and Johnson, 1994). Elimination of one of the protein kinases active in one MAPK pathway does not affect the function of the other pathway.

The plant responses induced by either fenthion or avirulent *P. s. tomato* strains are macroscopically similar. It is tempting to speculate that fenthion sensitivity mimics the HR. We are currently testing this hypothesis by examining histochemical and molecular phenomena correlated with an HR (Dietrich et al., 1994). Another piece of evidence to support the hypothesis that the Fen gene is involved in a signal transduction leading to an HR comes from the finding that expression of the *Fen* gene in *N. tabacum* and *N. clevelandii* produces a fenthion-independent necrotic HR-like response. This phenomenon is not observed upon expression of *Pto* or *Fen* kinase mutants in these plants. According to a recent proposal (Greenberg et al., 1994), it is possible that these two *Nicotiana* spp lack a Fen-specific, negative regulator. Arabidopsis accelerated death (acd) mutants spontaneously develop HR-like lesions and display characteristics associated with the HR; these researchers propose that the *acd* genes are negatively regulated. The availability of both fenthion and the *Pto*, *Fen*, and *avrPto* genes provides an ideal opportunity to further dissect signal transduction pathways that result in the production of HR-like lesions.

Systemic infection of tomato plants with PVX:Pto did not result in a *P. s. tomato* resistant phenotype. The lack of resistance may be associated with the uneven distribution of *Pto* gene expression throughout the leaf mesophyll. In another recent study, tomato plants containing the disease resistance gene * Cf-9* were infected with a PVX derivative carrying the corresponding avirulence gene *avr9* (Hammond-Kosack et al., 1995). Expression of the *avr9* gene from the virus genome induced a necrotic response in tomato, but this necrotic response did not arrest spread of the virus. Several explanations were given for the inability to generate effective resistance using PVX as a vector for gene expression. One of these explanations, which could also account for our results, is that PVX may replicate and spread through a plant cell lineage in which a defense response cannot be manifested (Hammond-Kosack et al., 1995). Alternatively, it could be possible that *Pto* expression driven from the coat protein promoter of PVX resulted in too high titers of Pto. Perhaps, successful elicitation requires a certain ratio of elicitor to receptor.

We showed that PVX insertion derivatives are able to spread in tomato plants and can express foreign genes in systemically infected leaf tissue. Expression was correlated with function in the case of Fen but not with Pto. Expression patterns were not uniform across the area of a leaflet. This may have been a result of genomic instability of PVX derivatives in tomato. Instability has been described previously for PVX derivatives in *N. clevelandii* by Chapman et al. (1992); they demonstrated the presence of both wild-type PVX:Gus and deletion mutants in *N. clevelandii*. The deletion mutants are similar in size to unmodified PVX, suggesting that the deletions may have occurred by homologous recombination between the duplicated promoter sequences. We showed that the PVX system is useful for rapid structure/function analyses of genes such as *Fen* in tomato. The ability to rapidly ascertain the phenotype of a mutated protein will allow us to test additional mutations in the *Fen* gene. These experiments may provide useful clues in understanding the mode of action of the *Fen* gene.

**METHODS**

**Plant Materials, Potato Virus X Infection, Fenthion Assay, and *Pseudomonas syringae pv tomato* Inoculation**

The near-isogenic *Lycopersicon esculentum* cv. Rio Grande 76R and 76S were obtained from Peto Seed Company (Woodland, CA). Tomato and tobacco plants were routinely grown in a temperature-controlled (average temperature 68°C) room in the greenhouse in clay pots in standard potting soil. Two-week-old tomato seedlings or four-week-old tobacco plants (*Nicotiana tabacum* and *N. clevelandii*) were infected with in vitro transcripts of potato virus X (PVX) or PVX derivatives (Chapman et al., 1992). To assay for susceptibility or resistance to *P. s. tomato* race 1 strain T1 carrying the avirulence gene *avrPto* (Salmeron and Staskawicz, 1993), leaves of tomato plants were either dipped into a suspension containing 10⁶ colony-forming units (cfu) of this pathogen and 0.03% Silwet L77 (Osi Specialties, Siersvilles, WV) or inoculated with 10⁶ cfu of T1(*avrPto*) by pressure infiltration (Salmeron et al., 1994). Reactions were assayed 4 days after *P. s. tomato* treatment. To assay tomato plants for sensitivity or insensitivity to fenthion, shoots were immersed in a fenthion solution (Baytex 4; Mobay Chemicals, Kansas City, MO) at a concentration of 2 mL/L in water containing 0.03% Silwet L77. Fenthion reactions were scored 4 days later as sensitive (displaying dark necrotic specks) or insensitive (no reaction) as described previously (Carland and Staskawicz, 1993).

**Analysis of β-Glucuronidase Activity**

Histochemical analysis of β-glucuronidase (Gus) activity with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid was performed as described previously (Jefferson, 1987).

**Agrobacterium-Mediated Transformation**

Tomato plants were transformed as described by McCormick et al. (1986).
Construction and Screening of a Tomato cDNA Library

To clone members of the Pto gene family, leaves of 76R plants were vacuum infiltrated with Tl(avrPto) at 2.5 x 10^7 cfu/mL (Ronald et al., 1992). After 6 hr, the infiltrated leaves were harvested and used to construct a cDNA library in the Uni-ZAP vector with a ZAP-cDNA synthesis kit (Stratagene). The primary library consisted of 3 x 10^6 plaque-forming units (pfu). Following amplification, 1.2 x 10^6 pfu were screened with a genomic DNA fragment homologous to Pto (C.M.T. Rommens, J.M. Salmeron, and B.J. Staskawicz, unpublished results). Phagemids containing cloned inserts were recovered from λ vectors according to the manufacturer's instructions.

Polymerase Chain Reactions and Primers

Polymerase chain reactions (PCRs) were performed in 100-µL volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCI, 2 mM MgCl2, 0.01% gelatin, 0.005% Tween 20, 0.005% Nonidet P-40, and 5 units of Taq DNA polymerase using the following profile: 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 25 cycles. The following primers were used:

P1, 5'-TCGATATCGATGGGAAGCAAGTATTCTAAGGC-3';
P2, 5'-AACATCCAG-3';
P3, 5'-GCCATATCTCTAGAATGCAGTTATACATCGTGA-3';
P4, 5'-TAAGAATGCAGTTATACATCGTGA-TGCTAAGTGAAGAACC-3';
P5, 5'-ACTCATGTAGCCTCAGCTAC-3';
P6, 5'-AGATATCTGAGAG-3';
P7, 5'-GATATCGTCGACTCAAGAAATGCAGTTATACATCGTGA-TGCTAAGTGAAGAACC-3';
P8, 5'-CGCCGAT-3';
P9, 5'-TCGATATCGATGGGAAGCAAGTAT-3';
P10, 5'-AACATCCAG-3'.

Construction and Transcription of PVX Derivatives

The sequence of the Pthl cDNA was used to design primers introducing a Clal restriction site at the first start codon and an EcoRV site 200 bp downstream from the stop codon. The 1.2-kb amplification product was digested with Clal and EcoRV and ligated into the PVX vector pPC2S, a derivative of pTXAGC3A (Hammond-Kosack et al., 1995), to give PVX:Pthl. PVX:Pto. pBlue:Pthl and pBlue:Pto were then digested with Clal and EcoRV and ligated into the PVX vector pPC2S, a pTXAGC3A derivative (Hammond-Kosack et al., 1995), to give PVX:Pthl and PVX:Pto.

In addition, mutant derivatives of Pto and Pthl were created. A myristoylation-minus Pthl mutant was obtained using primers P2 and P8 (introducing a GGA-to-AGA change that abolishes the putative myristoylation site at the N terminus). Pto and Pthl kinase mutants were created using PCR mutagenesis to replace Asn-167 by a Thr residue and Lys-69 by an Asn residue, respectively. The PCR product amplified from pBlue:Pthl with primers P4 (introducing an AAC-to-ACC change) and P2 was digested with BsmI and StuI; the resulting 0.24-kb fragment was used to replace the wild-type fragment of pBlue:Pthl to produce pBlue:Pthl(K167T). Primers P1 and P5 were used to amplify a 0.21-kb product from pBlue:Pto that was digested with Rsal and used to produce pBlue:Pto(K69N).

All constructs were verified by either single-stranded sequencing of plasmid DNA using Sequenase Version 2.0 (United States Biochemical Corp.) or double-stranded sequencing using PRISM ReadyReaction DyeDeoxy cycle sequencing according to the directions of the manufacturer (Applied Biosystems, Foster City, CA). To assay the effect of the mutation on activity, the mutant derivatives were cloned into pTXAGC3A (Hammond-Kosack et al., 1995).

Reverse Transcription-PCR

To confirm the presence of intact inserts once the PVX-based constructs were inoculated into plants, RNA from systemically infected leaves (i.e., uninoculated leaves) was analyzed by reverse transcription–PCR (RT-PCR; Simpson et al., 1992). PVX-specific primers P6, flanking the unique EcoRV site of pPC2S, and P7, flanking the unique ClaI site of pPC2S, were used.

Fusion Proteins and Kinase Assays

To obtain Pthl and Pto protein fusions with glutathione S-transferase (Gst), 1.2-kb SalI-NotI fragments of pBlue:Pthl and pBlue:Pto, respectively, were ligated into pGEX-5X-2 (Pharmacia Biotech). Pthl and Pto kinase mutant genes present in pBlue:Pthl(N167T) and pBlue:Pto(K69N) were cloned into pGEX-5X-2 as recommended by the manufacturer (Pharmacia Biotech). Kinase assays were performed as described by Goring and Rothstein (1992).

ACKNOWLEDGMENTS

We thank Kathy Swords, Maureen Whalen, Andy Jackson, Allan Shapiro, and Tom Tai for helpful comments on the manuscript; Greg Martin for communicating results before publication; Doug Dahlbeck and Barbara Rotz for expert assistance; Bradley Hall for plant transformations; Steve Scofield and John Gardner for constructing primers; and Herman Scholthof for advice on virus assays. C.M.T.R. was supported by a post-doctoral fellowship of the Netherlands Organization for Scientific Research and a fellowship from the European Molecular Biology Organization. J.M.S. was supported by a grant from Zeneca Seeds (Bracknell, UK). Part of this work was supported by the National Science Foundation Center for Engineering Plants for Resistance Against Pathogens (CEPRAP).

Received October 20, 1994; accepted January 12, 1995.

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Characterization of a Pto Homolog Using PVX


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*Plant Cell* 1995;7:249-257

DOI 10.1105/tpc.7.3.249

This information is current as of June 21, 2017