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Rapid Communication

The *Pto* Bacterial Resistance Gene and the *Fen* Insecticide Sensitivity Gene Encode Functional Protein Kinases with Serine/Threonine Specificity[1]

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The catalytic activity and amino acid specificity of the tomato Pto and Fen kinases were investigated. The *Pto* and *Fen* genes were fused to the carboxyl terminus of the maltose-binding protein and expressed in *Escherichia coli*. Incubation of the purified fusion proteins with [γ-^{32}P]ATP in an in vitro assay showed that both proteins were capable of autophosphorylation. Mutant fusion proteins in which the conserved lysine residue of subdomain II was changed to a glutamine were unable to autophosphorylate. Phosphoamino analysis of the active fusion proteins indicated that both kinases phosphorylate serine and threonine residues but not tyrosine.

Resistance of tomato to *Pseudomonas syringae* pv *tomato* strains that express the avirulence gene *avrPto* is conferred by the *Pto* gene (Martin et al., 1993). *Pto* is a member of a small gene family that is clustered on chromosome 5. Another member of this family, *Fen*, confers sensitivity to an organophosphorous insecticide, fenthion (Martin et al., 1994). The deduced proteins encoded by *Pto* and *Fen* contain the hallmarks of protein kinases, namely 15 conserved amino acid residues located in 11 subdomains. Eukaryotic protein kinases are broadly classified into two groups based on their ability to phosphorylate either Ser/Thr residues or Tyr residues (Hanks et al., 1988; Hanks and Quinn, 1993). In common with several other plant protein kinases, *Pto* and *Fen* contain residues in subdomains VIb and VIII that indicate specificity for phosphorylation of Ser and Thr (Martin et al., 1993, 1994). To extend our understanding of the signal transduction pathway(s) in which Pto and Fen participate, we investigated the activity of these kinases by using an in vitro phosphorylation assay and analyzing their amino acid specificity.

Genes encoding putative protein kinases have been isolated from a variety of plant species, including *Arabidopsis thaliana*, bean, maize, rice, tobacco, tomato, pea, petunia, and two *Brassica* sp. (Lawton et al., 1989; Walker and Zhang, 1990; Stein et al., 1991; Goring and Rothstein, 1992; Hirayama and Oka, 1992; Lin et al., 1992; Tobias et al., 1992; Chang et al., 1993; Kieber et al., 1993; Martin et al., 1993, 1994; Ali et al., 1994; Dwyer et al., 1994; Horn and Walker, 1994; Ito et al., 1994; Mu et al., 1994; Zhao et al., 1994). The roles of only a few of these kinases in cellular processes are known. CTR1 encodes a putative Ser/Thr kinase, which, by genetic analysis, is thought to be a negative regulator of the ethylene-response pathway in Arabidopsis. Another kinase involved in ethylene recognition and signal transduction is ETR1, although this protein is unique in that it resembles a His-type kinase similar to the sensor component of prokaryotic two-component systems (Chang et al., 1993). Two kinases, SRK6 and SRK910, have been identified that are associated with sporophytic self-incompatibility in *Brassica oleracea* and *Brassica napus*, respectively (Stein et al., 1991; Goring and Rothstein, 1992). Both *Brassica* kinases have been shown to have Ser/Thr specificity (Goring and Rothstein, 1992; Stein and Nasrallah, 1993). Protein kinases with demonstrated Ser/Thr specificity have also been characterized from rice (Zhao et al., 1994), tobacco (Ito et al., 1994), and *A. thaliana* (Chang et al., 1992; Horn and Walker, 1994). Two kinases are known to have dual specificity, phosphorylating both Ser/Thr and Tyr residues (Hirayama and Oka, 1992; Ali et al., 1994); whereas a kinase from *Petunia inflata* was shown to phosphorylate Ser and Tyr residues (Mu et al., 1994). Despite the isolation of numerous plant protein kinases, in most cases, little is known of their specific roles in plant signal transduction.

We report here that recombinant fusion proteins of both Pto and Fen are able to autophosphorylate specifically on Ser and Thr residues. Furthermore, mutation of the nucleotides that encode a conserved Lys residue at an ATP-binding site (subdomain II) abolishes kinase activity. These studies lay the foundation for investigating possible substrates of the *Pto/Fen* kinases using both biochemical and molecular approaches.

MATERIALS AND METHODS

Construction of Pto and Fen Fusion Proteins

PTC1 is a plasmid containing the 1.2-kb CD127 cDNA insert encoding *Fen* cloned into vector pCDNAII (Invitrogen Co., San Diego, CA; Martin et al., 1994). Similarly, PTC3 contains the 2.4-kb cDNA insert encoding *Pto* cloned

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Abbreviation: ORF, open reading frame.

into pCDNAII (Martin et al., 1993). Oligonucleotide primers used to develop the fusion proteins and to mutagenize the Lys codon are as follows: YTL3, 5′-GGG AAT TCA TGG GAA GCA AGT ATT C-3′; YTL4, 5′-GGG ATA TCA TGG GAA GCA AGT ATT C-3′; YTL5, 5′-CCC TGC AGT GAA AGA AGG ATC CAC AG-3′; YTL6, 5′-AAG GTC GCC CTG CAG AAG CAT AAA CCT GAG-3′; YTL7, 5′-AAG GTG GCC CTG CAG AGG CGT ACA CCT GAG-3′; SP6, 5′-ATT TAG GTG ACA CTA TAG-3′. The entire 963-bp ORF1 of *Pto* (Martin et al., 1993) was amplified from PTC3 using primers YTL3 and YTL5. The resulting 1002-bp fragment was gel purified (Geneclean; Bio101, La Jolla, CA), digested with *Eco*RI and *Pst*I, and subcloned into the corresponding sites in the bacterial expression vector pMAL-c2 (New England Biolabs) to develop an in-frame fusion plasmid, pMBP-Pto. The entire 954-bp ORF of *Fen* was amplified from PTC1 using primers YTL4 and SP6. The PCR product was digested with *Eco*RV and *Hin*dIII to obtain a 1082-bp fragment, and this fragment was subcloned into the *Xmn*I/*Hin*dIII sites of pMAL-c2 to yield an in-frame fusion plasmid, pMBP-Fen.

Mutant fusion proteins in which the Lys at position 69 in both Pto and Fen (Martin et al., 1993, 1994) was replaced with Gln were obtained by site-directed mutagenesis using the method described by Jung et al. (1992). For the Pto construct, phosphorylated mutagenic primer YTL7 was annealed to single-stranded PTC3, polymerized, and ligated according to the method of Jung et al. (1992). YTL7 encompasses kinase subdomain II and introduces two nucleotide changes that replace the Lys with a Gln residue upon translation. Confirmed mutagenized plasmid DNA was digested with *Nsi*I and *Xho*I, enzymes that flanked the mutagenized region. This yielded a 390-bp fragment containing the converted Gln residue. The fragment was subcloned into the corresponding *Nsi*I/*Xho*I sites in digested pMBP-Pto. The final construct was called pMBP-Pto(K69Q).

A mutagenized Fen fusion protein was produced using the same approach with slight modifications. Phosphorylated, mutagenic primer YTL6 was annealed, polymerized, and ligated to pMBP-Fen as described above and by Jung et al. (1992). YTL6 encompasses subdomain II and introduces two nucleotide changes that result in the replacement of the Lys with a Gln residue. The mutagenized construct was called pMBP-Fen(K69Q). The fusion constructs were transformed into a protease-deficient bacterial strain, PR745 (*lon*$^-$; New England Biolabs) for use in subsequent experiments.

Expression and Purification of Fusion Proteins

Overnight cultures of PR745 containing the fusion constructs were diluted 1:10 in terrific broth (Sambrook et al., 1989) with 1 mM $MnCl_2$ plus ampicillin and grown for 1.5 h at 37°C. Expression of the fusion proteins was induced by adding 0.05 mM isopropyl β-thiogalactoside and incubating the bacteria for 45 min at 25°C. Cells were harvested in isolation buffer (Tris-buffered saline: 10 mM Tris, pH 7.3, 150 mM NaCl, 1 mM DTT, 1 mM PMSF) and sonicated, and the solubilized fusion protein was affinity purified by binding to amylose resin on ice for 20 min as described by Maina et al. (1988) and Chang et al. (1992). The resin was washed three times with isolation buffer and once with kinase buffer (50 mM Tris, pH 7.0, 1 mM DTT, 10 mM $MnCl_2$).

Autophosphorylation Assay and Phosphoamino Analysis of Fusion Proteins

For the kinase assays, 20 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) was added directly to the fusion proteins bound to amylose resin in kinase buffer (Chang et al., 1992). The mixture was incubated for 15 min at room temperature, and the resin was washed twice with 10 mM EDTA in Tris-buffered saline. Protein was eluted from the resin with SDS loading buffer containing 10 mM EDTA, boiled for 5 min, and resolved by SDS-PAGE. The gel was stained with Coomassie brilliant blue G-250, dried, and subjected to autoradiography.

For phosphoamino acid analysis, each autophosphorylated fusion protein labeled with [γ-^{32}P]ATP was subjected to SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore). A small piece of membrane containing the fusion protein was placed in 200 μL of constantly boiling (110°C) 6 N HCl for 1 h. The supernatant was removed and evaporated with a vacuum centrifuge. The residue was resuspended in 3 μL of buffer (pH 3.5; 50:5:45, glacial acetic acid:pyridine:double-distilled H_2O; Boyle et al., 1993; Van der Geer et al., 1993) that contained 1 μg each of phosphoserine and phosphothreonine and 2 μg of phosphotyrosine as standards. The entire sample was loaded onto a cellulose-backed TLC plate (EM Separations, Gibbstown, NJ) and subjected to electrophoresis at 1200 V for 1 h. The plate was sprayed with ninhydrin (0.5% in acetone) to visualize the standards and then subjected to autoradiography.

RESULTS

The complete ORFs of *Pto* and *Fen* were expressed as fusion proteins in *Escherichia coli* by cloning cDNA inserts into the vector pMAL-c2. Expression of pMBP-Pto and pMBP-Fen in *E. coli* was induced with isopropyl β-thiogalactoside and yielded fusion proteins with the expected masses of approximately 78 kD (MBP-Pto, 78.3 kD; MBP-Fen, 77.8 kD; Fig. 1A, lanes 1 and 3, respectively). In addition, antisera prepared against synthetic peptides of Pto and Fen detected the expressed fusion proteins on a western blot (Y.-T. Loh and G.B. Martin, unpublished results).

Activity of the fusion proteins was shown using an in vitro kinase assay. The fusion proteins MBP-Pto and MBP-Fen were purified by binding to amylose resin and incubated in kinase buffer with [γ-^{32}P]ATP. After the reaction, the amylose beads were washed to remove excess isotope, and the protein was eluted from the beads and resolved by SDS-PAGE. Autoradiography revealed radiolabeled bands at locations corresponding to the purified fusion proteins (Fig. 1B, lanes 1 and 3).

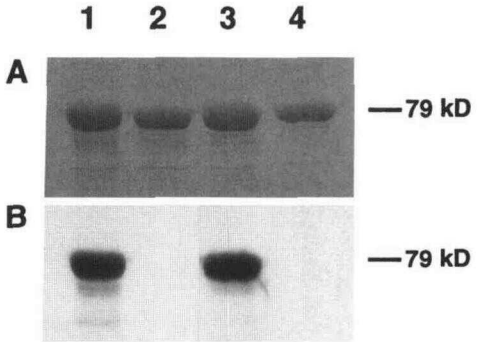

Figure 1. Expression of fusion proteins in *E. coli* and in vitro kinase assays. A, Gel of purified fusion proteins stained with Coomassie blue. Solubilized proteins were affinity purified by binding to amylose resin, subjected to the kinase assay, eluted from the beads, and resolved on SDS-PAGE. Shown are the fusion proteins MBP-Pto, MBP-Pto(K69Q), MBP-Fen, and MBP-Fen(K69Q) (lanes 1–4, respectively). B, Autoradiograph to detect the autophosphorylation of the proteins shown in A. The affinity-purified fusion proteins were tested for their ability to autophosphorylate by the addition of [γ-^{32}P]ATP. Lanes 1 and 3, Fusion protein expressed by pMBP-Pto and pMBP-Fen, respectively. Lanes 2 and 4, Fusion proteins expressed by the mutagenized constructs pMBP-Pto(K69Q) and pMBP-Fen(K69Q). Standards from Bio-Rad were used to estimate protein sizes. Gel was exposed to film for 24 h (lanes 1 and 2) or 48 h (lanes 3 and 4).

To verify that phosphorylation of the fusion proteins was not caused by contaminating bacterial proteins, mutant constructs of the proteins pMBP-Pto(K69Q) and pMBP-Fen(K69Q) were generated by site-specific in vitro mutagenesis, in which the invariant Lys residue of subdomain II was converted to Gln. The invariant Lys residue of subdomain II is the best characterized catalytic residue in protein kinases and is known to be directly involved in phosphotransfer (Hanks et al., 1988). When the altered fusion proteins were expressed in *E. coli*, they produced proteins of similar sizes as the wild-type constructs (Fig. 1A, lanes 2 and 4). In vitro kinase assays of the mutant fusion proteins showed that they did not become phosphorylated (Fig. 1B, lanes 2 and 4). We conclude from these experiments that both Pto and Fen possess intrinsic kinase activity.

Both Pto and Fen contain amino acids in subdomains VIb and VIII, which indicate they may have specificity for Ser/Thr residues (Fig. 2; Martin et al., 1993, 1994). To test the specificity of the Pto and Fen kinases, we analyzed the composition of autophosphorylated amino acids by TLC (Fig. 3). Affinity-purified ^{32}P-labeled fusion proteins MBP-Pto and MBP-Fen were isolated from SDS-PAGE gels and hydrolyzed into their constituent amino acids by treatment with boiling HCl. The hydrolysate was fractionated by electrophoresis on a one-dimensional, thin-layer chromatographic plate. Standards of phosphoserine, phosphothreonine, and phosphotyrosine were included with the radiolabeled samples to monitor migration of these amino acids. This analysis showed that radiolabeled amino acids derived from the hydrolyzed MBP-Pto and MBP-Fen proteins co-migrated with the phosphoserine and phosphothreonine standards (Fig. 3). Ser residues were phosphorylated significantly less than Thr, although the biological significance of this, if any, is unknown. Although Pto and Fen both specifically phosphorylate Ser and Thr, Tyr residues were not phosphorylated.

DISCUSSION

In vitro autophosphorylation assay of bacterially expressed proteins is a commonly used method to study potential activity of protein kinases and offers the advantage that it requires no knowledge of the endogenous substrate (Wang et al., 1982; Tan and Spudich, 1990; Chang et al., 1992; Stein and Nasrallah, 1993). We have shown, using an in vitro kinase assay, that both Pto and Fen are functional protein kinases. In addition, we demonstrated that replacement of the conserved Lys residue in the phosphotransfer site (subdomain II) with Gln abolishes kinase activity.

Based on the presence of key invariant amino acids in kinase subdomains VIb and VIII, we postulated that Pto and Fen would have Ser/Thr specificity (Martin et al., 1993, 1994). Nevertheless, it is important to demonstrate this specificity, because in some cases proteins with these invariant residues are known to have dual specificity (Fig. 2; Hirayama and Oka, 1992; Mu et al., 1994). Our analysis of the composition of autophosphorylated Pto and Fen indicates that they contained only phosphorylated Ser and Thr residues. These data thus support the hypothesis that Pto and Fen are Ser/Thr kinases that participate in a phosphorylation cascade which eventually leads to resistance against the bacterial pathogen *P. syringae* pv *tomato* and to fenthion sensitivity, respectively.

The development of necrotic lesions on Fen-containing tomato leaves in response to fenthion exposure is reminiscent of a hypersensitive resistance response often observed

Subdomain:	VIB						VIII								
Consensus:	D	L	K	P	E	N	G	T/S	X	X	Y/F	X	A	P	E
Fen	D	V	K	C	T	N	G	N	I	G	Y	I	A	P	E
Pto	D	V	K	S	I	N	G	T	L	G	Y	I	D	P	E
RLK5	D	V	K	S	S	N	G	S	C	G	Y	I	A	P	E
NPK15	D	L	K	S	A	N	G	T	Y	G	Y	I	D	P	V
TMK1	D	L	K	P	S	N	G	T	F	G	Y	L	A	P	E
SRK6	D	L	K	V	S	N	G	T	Y	G	Y	M	S	P	E
SRK910	D	M	K	V	S	N	G	T	Y	G	Y	M	S	P	E
OsPK10	D	I	K	P	E	N	G	T	R	G	Y	L	A	P	E
APK1(s/t/y)	D	F	K	T	S	N	G	T	H	G	Y	A	A	P	E
ADK1(s/t/y)	D	I	K	P	D	N	G	T	A	R	Y	A	S	V	N
PRK1(s/y)	H	L	K	S	S	N	L	L	V	A	Y	K	A	P	E

Figure 2. Alignment of subdomains VIb and VIII from plant protein kinases with demonstrated Ser/Thr or dual specificity. A consensus sequence derived from an analysis of 75 Ser/Thr kinases isolated from various organisms is shown (Hanks and Quinn, 1993). Residues indicative of Ser/Thr specificity are shaded. All kinases have Ser/Thr specificity except APK1, ADK1, and PRK1, which have amino acid specificities as indicated in parentheses (Ser [s], Thr [t], and Tyr [y]). References to sequences are: Pto, Martin et al., 1993; Fen, Martin et al., 1994; RLK5, Horn and Walker, 1994; NPK15, Ito et al., 1994; TMK1, Chang et al., 1992; SRK6, Stein et al., 1991; SRK910, Goring and Rothstein, 1992; OsPK10, Zhao et al., 1994; APK1, Hirayama and Oka, 1992; ADK1, Ali et al., 1994; PRK1, Mu et al., 1994.

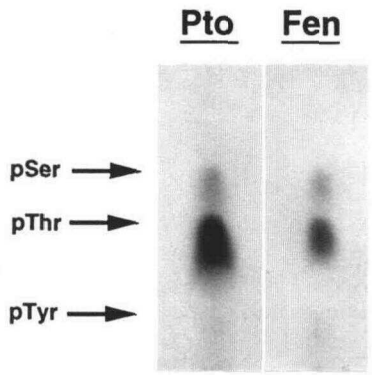

Figure 3. Phosphoamino analysis of the ^{32}P-labeled autophosphorylated MBP-Pto and MBP-Fen fusion proteins. Radiolabeled, autophosphorylated proteins were transferred to a polyvinylidene difluoride membrane, eluted, and hydrolyzed to compositional amino acids as described in "Materials and Methods." Samples were separated by one-dimensional TLC. The locations of phosphoamino standards of Ser, Thr, and Tyr as determined by ninhydrin are indicated. The TLC plate was exposed to film for 4 d (Pto) or 6 d (Fen).

in incompatible plant-pathogen interactions (Martin et al., 1994). This similarity of phenotype, together with the structural similarity of Pto and Fen, raises the interesting possibility that the unknown bacterial signal molecule produced by avirulent *P. syringae* pv *tomato* may be structurally analogous to fenthion (Martin et al., 1994). The mechanism(s) whereby Pto and Fen recognize a signal from an avirulent bacterium or the fenthion molecule is unknown. It is possible that these kinases interact with an extracellular receptor that has previously been activated by binding a race-specific elicitor molecule from the bacterial pathogen or the fenthion molecule. In this regard, a potential myristoylation site present in both kinases may serve to position the proteins at the plasma membrane near a receptor. Alternatively, a pathogen signal molecule (or fenthion) may interact directly with Pto (or Fen) and enhance the phosphorylation capability of the kinases. To test this latter hypothesis, we added fenthion directly to in vitro kinase assays that contained purified Fen protein (Y.-T. Loh and G.B. Martin, unpublished results). However, in these preliminary experiments we observed no increase in Fen autophosphorylation. It remains a possibility that fenthion is metabolized to another compound in planta, and it is this molecule that interacts with Fen. Alternatively, fenthion may enhance phosphorylation of a specific substrate.

These results lay the foundation for future studies of the signal transduction pathway(s) in which Pto and Fen participate. For example, we are currently using the yeast two-hybrid system and other techniques to identify proteins that may physically interact with Pto and Fen. The possibility that some of these proteins may be substrates will be tested using the in vitro kinase assay and fusion proteins described here.

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