Biochemical Properties of Two Protein Kinases Involved in Disease Resistance Signaling in Tomato*

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Guido Sessa, Mark D’Ascenzo, Ying-Tsu Loh, and Gregory B. Martin‡

From the Department of Agronomy, Purdue University, West Lafayette, Indiana 47907-1150

In tomato plants, resistance to bacterial speck disease is mediated by a phosphorylation cascade, which is triggered by the specific recognition between the plant serine/threonine protein kinase Pto and the bacterial AvrPto protein. In the present study, we investigated in vitro biochemical properties of Pto, which appears to function as an intracellular receptor for the AvrPto signal molecule. Pto and its downstream effector Pti1, which is also a serine/threonine protein kinase, were expressed in Escherichia coli as maltose-binding protein and glutathione S-transferase fusion proteins, respectively. The two kinases each autophosphorylated at multiple sites as determined by phosphopeptide mapping. In addition, Pto and Pti1 autophosphorylation occurred via an intramolecular mechanism, as their specific activity was not affected by their molar concentration in the assay. Moreover, an active glutathione S-transferase-Pto fusion failed to phosphorylate an inactive maltose-binding protein-Pto(K69Q) fusion excluding an intermolecular mechanism of phosphorylation for Pto. Pti1 phosphorylation by Pto was also characterized and found to occur with a $K_m$ of 4.1 $\mu$M at sites similar to those autophosphorylated by Pti1. Pto and the product of the recessive allele pto phosphorylated Pti1 at similar sites, as observed by phosphopeptide mapping. This suggests that the inability of the kinase pto to confer resistance to bacterial speck disease in tomato is not caused by altered recognition specificity for Pti1 phosphorylation sites.

Higher plants have evolved the ability to recognize and resist invading pathogens by the activation of defense mechanisms that inhibit pathogen growth and movement in the plant (1). In many plant-pathogen interactions, the rapid activation of the defense response is mediated by a specific recognition event involving the product of an avirulence (avr) gene in the pathogen and the corresponding resistance (R) gene in the plant (2).

Proteins encoded by $R$ genes are postulated to function as receptors that bind the cognate avr gene product and activate defense mechanisms. Several $R$ genes have been isolated to date, and structural characteristics of their encoded proteins support the proposed receptor function (3). Most $R$ genes encode cytoplasmic or transmembrane proteins containing a region of leucine-rich repeats of variable length and content, which might be involved in protein-protein interactions (4). Some leucine-rich repeat-containing $R$ proteins, including those encoded by the tobacco $N$, the flax $L_6$, and the Arabidopsis $RPP5$ genes, also contain a region of homology with the cytoplasmic domains of the Drosophila Toll and the mammalian interleukin-1 receptor proteins (4).

Another class of $R$ genes is represented by Pto, which encodes a tomato serine/threonine protein kinase and confers resistance specifically to the bacterial pathogen Pseudomonas syringae pv. tomato expressing the avirulence gene avrPto (5). Pto does not share motifs with other resistance genes, except for the kinase domain, which is also present in the product of the rice gene Xo21, conferring resistance to the bacterial pathogen Xanthomonas oryzae pv. oryzae (6). Recent studies have shown, using the yeast two-hybrid system, that a direct interaction occurs between the product of the tomato Pto resistance gene and the product of the P. syringae pv. tomato avrPto gene (7, 8). Mutations of Pto or AvrPto that interfere with this interaction in yeast also abolish disease resistance in plants. In addition, for resistance to take place, Pto and AvrPto must be present simultaneously inside the plant cell, strongly suggesting that the Pto-AvrPto recognition is an intracellular event. AvrPto and other bacterial avr gene products are thought to be delivered into the plant cell by a type III secretion system encoded by the bacterial pathogen (9). In fact, a set of genes in P. syringae shows similarity to genes encoding components of the type III secretion system of mammalian pathogens, and is required both for resistance and pathogenicity (10).

The recognition event between the tomato Pto kinase and the bacterial AvrPto protein initiates a signal transduction pathway that involves downstream effectors and ultimately leads to disease resistance. The Prf gene is required for Pto-mediated resistance and encodes a protein with a leucine zipper, a nucleotide binding site, and leucine-rich repeats, common motifs in other resistance gene products (11). However, the role of Prf in the Pto pathway remains unclear. Other putative effectors were identified by their specific interaction with the Pto kinase in the yeast two-hybrid system (12, 13). Among them are Pti1, a serine/threonine protein kinase that is specifically phosphorylated in vitro by Pto and is involved in the hypersensitive response (12), and Pti4, Pti5, and Pti6, putative transcription factors that are similar to the tobacco ethylene-responsive element-binding proteins (13, 14).

In mammals, autophosphorylation activity plays a central role in the regulation of receptor tyrosine kinases (15). Hormones, or growth and differentiation factors, bind to receptors with tyrosine kinase activity and induce conformational alterations in the receptor extracellular domains causing oligomerization, polyphosphorylation, and activation of intracellular signaling pathways.
Activities of Pto and Pti1 Protein Kinases

The stained gel was dried and exposed to x-ray film. To determine \(V_{\text{max}}\) and \(K_m\) values of the MBP-Pto fusion for its substrate GST-Pti1(K96N) fusion protein, 0.5 \(\mu\)g of MBP-Pto was incubated at room temperature with different amounts of GST-Pti1(K96N) in 20 \(\mu\)l of kinase buffer containing 2 \(\mu\)Ci of \([\gamma^{32}\text{P}]\text{ATP}\) (6000 Ci/mmol; Amersham Pharmacia Biotech). The range of GST-Pti1(K96N) concentrations tested was from 0.72 to 17.9 \(\mu\)M. Reactions were stopped after 10 min by adding EDTA to a final concentration of 10 mM. At this reaction time, phosphate incorporation was found to be linear for the highest substrate concentration used in the experiment. Proteins were then fractionated by SDS-PAGE, stained by Coomassie Brilliant Blue R250, and analyzed by Instant Imager (Packard Corp.).

**RESULTS**

**Pto and Pti1 Autophosphorylation Occur through an Intramolecular Mechanism**—The Pto and Pti1 proteins are involved in resistance to bacterial speck disease in tomato plants and have been shown previously to be active serine/threonine kinases when tested in vitro (12, 19). To further characterize their kinase activity, we tested whether Pto and Pti1 autophosphorylation mechanisms are intramolecular (first order with respect to enzyme concentration) or intermolecular (second order with respect to enzyme concentration). Pto and Pti1 were expressed in bacteria as MBP (MBP-Pto) and GST (GST-Pti1) fusion proteins, respectively, and the effect of various molar concentrations on the autophosphorylation reaction was studied. As shown in Figs. 1A and 2A, the rate of autophosphorylation was linear with respect to enzyme concentration for both MBP-Pto and GST-Pti1. In addition, the phosphate incorporation per molecule was constant when MBP-Pto concentration in the reaction varied by 60-fold (Fig. 1B). Similarly, the phosphate incorporation per GST-Pti1 molecule varied by only 1.7 when the enzyme concentration in the reaction varied by 140-fold (Fig. 2B). Finally, the van't Hoff plot of autophosphorylation (logarithm of phosphorylation rate versus logarithm of enzyme concentration), whose slope indicates the order of the reaction, had a slope of 1.11 ± 0.038 and 0.90 ± 0.010 for MBP-Pto and GST-Pti1, respectively (Figs. 1C and 2C). Taken together, these data indicate that both MBP-Pto and GST-Pti1 autophosphorylation occur predominantly via an intramolecular mechanism.

To provide further evidence for intramolecular autophosphorylation by Pto, we tested if an active GST-Pto fusion protein can phosphorylate an inactive MBP-Pto molecule in which the invariant lysine residue in kinase subdomain II was substi-
tuted by a glutamine (19). As shown in Fig. 3, the GST-Pto fusion protein was able to autophosphorylate and to phosphorylate its substrate Pti1 as observed previously (12). However, it failed to phosphorylate the inactive mutant protein MBP-Pto(K69Q), strongly supporting the notion that Pto autophosphorylation occurs through an intramolecular rather than intermolecular mechanism.

Kinetic Analysis of Pti1 Phosphorylation by Pto—The Pti1 protein kinase was shown previously to be specifically phosphorylated in vitro by the Pto protein kinase (12). To study the kinetics of this reaction, the initial velocity of the phosphorylation of a kinase-deficient mutant GST-Pti1(K96N) by Pto-MBP was analyzed at different substrate concentrations (Fig. 4). Nonlinear least squares fitting analysis of the data estimated $K_m$ and $V_{max}$ values for the GST-Pti1(K96N) substrate as $4.1 \pm 0.6 \mu M$ and $0.55 \pm 0.03$ nmol/min/mg, respectively.

Tryptic Phosphopeptide Mapping of Pto and Pti1—To investigate Pto and Pti1 autophosphorylation sites in more detail, MBP-Pto and GST-Pti1 fusion proteins were autophosphorylated in vitro and digested with trypsin. The tryptic digests...
were resolved horizontally by thin layer electrophoresis at pH 4.7, and vertically by ascending chromatography, as indicated in A. The origin is indicated by an asterisk in each plate.

recessive allele pto, which is a functional protein kinase but does not confer bacterial speck resistance to tomato plants (20). To compare the Pti1 autophosphorylation sites to those phosphorylated by Pto or pto, the kinase-deficient mutant GST-Pti1(K96N) was phosphorylated by an MBP-Pto or MBP-pto fusion proteins and then digested by trypsin. Phosphopeptide maps of the Pti1 digestion products revealed the presence of one major phosphorylated peptide, similar to that observed in Pti1 autophosphorylation reactions (Fig. 5, B–D). To test whether this major spot was derived from the same peptide phosphorylated by Pti1 autophosphorylation and by Pto and recessive pto phosphorylation, tryptic digests from the three reactions were fractionated by alkaline electrophoresis. As shown in Fig. 6, the main phosphorylated peptide in all the reactions showed the same molecular weight and charge characteristics, suggesting that the same phosphorylation site(s) may be utilized by the three enzymes. Similar minor spots were observed in the phosphotryptic maps of autophosphorylated Pti1 and of Pti1 phosphorylated by Pto or recessive pto (Fig. 5, B–D). Their intensity was variable in different experiments, and analysis of their sequence will be required to determine whether they represent phosphorylation sites, degradation products, or partial digests of the main phosphorylated peptide.

**DISCUSSION**

In this report, we characterized biochemical properties of two tomato serine/threonine protein kinases, Pto and Pti1, which are involved in the signaling pathway leading to resistance to the bacterial pathogen *P. syringae* pv. *tomato* expressing the *avrPto* gene. A specific recognition between the Pto kinase and the bacterial AvrPto protein occurs within the plant cell and triggers the activation of the pathway, defining the Pto kinase as an intracellular receptor or as part of a receptor complex (7, 8). In order to investigate the molecular mechanisms taking place in this interaction and the role of autophosphorylation in
Pto kinase activation, we first examined the molecular characteristics of Pto autophosphorylation in vitro. We found that Pto autophosphorylates at several sites in the protein via an intramolecular process that is not affected by protein concentration.

Induction of autophosphorylation activity by extracellular signals is well documented in mammals for receptors with tyrosine kinase activity (23). Ligand binding to the extracellular domain of receptor tyrosine kinases induces receptor dimerization. Dimerization in turn activates autophosphorylation of the receptor catalytic domain, which is mediated by an intramolecular mechanism of phosphorylation. Dimerization and intermolecular autophosphorylation of a nuclear serine/threonine kinase from Arabidopsis thaliana have been shown recently (24). However, the mechanism of activation for this protein kinase, essential for leaf and flower morphogenesis, is still unknown. In order to determine whether Pto dimerization and activation is plausible in the interaction between the signal molecule AvrPto and the Pto kinase, we tested in vitro the mechanism of Pto autophosphorylation. We found that Pto autophosphorylation occurs via an intramolecular reaction, making it unlikely that oligomerization is required for Pto activation.

Alternative modes of activation remain to be tested to elucidate the molecular mechanisms taking place during the Pto-AvrPto interaction. It is possible that AvrPto might activate Pto by causing conformational changes that possibly expose certain domains, which were not available previously, to autophosphorylation or to phosphorylation by an additional protein kinase. Such a mechanism occurs during activation of cyclin-dependent kinases (CDKs) by cyclins (25). The activity of CDK2, for example, which is involved in regulation of events in the eukaryotic cell cycle, is stimulated by a two-step mechanism of activation. First, the regulatory subunit cyclin A associates with CDK2, causing conformational changes in the kinase catalytic sites that make Thr-160 more accessible for phosphorylation by the CDK-activating kinase. Second, CDK-activating kinase phosphorylates Thr-160 in vitro determined full activation of CDK2 (26).

Even in such a scenario, autophosphorylation activity of Pto still may represent a prerequisite for the interaction with AvrPto. In fact, it has been observed that forms of Pto mutated in residues essential for kinase activity do not interact with AvrPto in the two-hybrid system (7, 8). The requirement of a phosphorylated residue for the interaction between a kinase protein and a regulatory subunit has been observed for the cyclic AMP-dependent protein kinase (27). Cyclic AMP-depend-
Activities of Pto and Pti1 Protein Kinases

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