The Pto Kinase of Tomato, Which Regulates Plant Immunity, Is Repressed by Its Myristoylated N Terminus*

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Specific recognition of the Pseudomonas syringae effector proteins AvrPto and AvrPtoB in tomato is mediated by Pto kinase resulting in induction of defense responses, including hypersensitive cell death via a signaling pathway requiring the nucleotide-binding leucine-rich repeats protein Prf. Pto is a myristoylated protein, and N-myristoylation is required for signaling. Here we demonstrated a role for N-myristoylation in controlling Pto kinase activity. A myristoylated peptide corresponding to Pto residues 2–10 significantly impaired the kinase activity of N-truncated Pto. We show that kinase inhibition was specific to the myristoylated form of the peptide and that free myristate supplied in trans was a potent suppressor of Pto kinase activity. Thus, myristate, but not Pto residues 2–10, contributes to suppression of kinase activity in vitro. Accordingly, elimination of the in vivo myristoylation potential of Pto de-repressed kinase activity. The increased potency of free myristate relative to the myristoylated N-peptide inhibitor suggested that the peptide moiety is antagonistic to repression by myristate. Suppression of related protein kinases by myristate declined with similarity to Pto, and the inhibitory activity could be attributed to hydrophobicity. We present evidence that inhibition of Pto by the myristoylated N-peptide is mediated through a previously identified surface regulatory patch. The data show a role for negative regulation of Pto by N-myristoylation, in addition to the previously demonstrated positive role, and are consistent with a model in which the acylated N terminus is sequestered in the catalytic cleft prior to release by Pto activation.

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3 The abbreviations used are: Avr, avirulence gene; CGF, constitutive gain-of-function; CoA, coenzyme A; GST, glutathione S-transferase; HA, hemagglutinin peptide; IMAC, immobilized metal ion (Ni²⁺) affinity chromatography; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; myr-CoA, myristate-CoA; myr-PKC, myristoylated PKC; p38, myristoylated N-terminal Pto peptide corresponding to residues 2–10; NBARC-LRR, nucleotide-binding leucine-rich repeats; NRP, negative regulatory patch; PKC, protein kinase C; R, resistance gene; SYMRK, symbiosis receptor kinase.
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activity. Abolishing myristoylation of the β1 subunit of AMP-activated protein kinase resulted in a significant increase in kinase activity (22). This is reminiscent of an autoinhibitory mechanism in the nonreceptor tyrosine kinase c-Abl, for which elimination of the myristoylation potential also de-represses kinase activity (23). In c-Abl, the myristate group is sequestered in a hydrophobic pocket within the kinase domain, inducing a bend within a C-terminal α-helix and promoting docking of domains distal to the c-Abl active site, thus locking the enzyme in a regulated, autoinhibited conformation (23, 24). Upon activation, removal of the myristate group from the hydrophobic pocket might mimic the unmyristoylated form, thus enhancing enzymic activity.

We recently identified a surface-exposed, hydrophobic region on Pto that confers negative regulation of Pto signaling (the negative regulatory patch (NRP) (25)). This surface is required for Pto-Avr interaction and spans the Pto catalytic cleft and adjacent residues. Mutations within the NRP result in CGF forms of Pto that elicit Avr-independent cell death in N. benthamiana. We proposed a model in which the NRP is normally occupied by a negative regulatory molecule that acts to repress Pto kinase activity. Avr binding displaces the repressor, allowing regulatory phosphorylation and a conformational change in the Pto tertiary structure leading to activation (25). The identity of the putative negative regulator is unknown.

In this paper we describe a role of the myristoylated Pto N terminus in suppressing Pto kinase activity in vitro. We show that myristate-modified peptides are potent inhibitors of the auto- and trans-phosphorylation activities of Pto. Free myristate severely inhibited kinase capability in a dose-dependent manner. Removal of the myristoylation potential of Pto expressed in planta resulted in stimulation of kinase activity relative to the wild type protein. We show that the inhibitory effect of the myristoylated N-peptide is mediated by a key residue within the NRP. Our data suggest that the myristoylated N terminus may be associated with the catalytic cleft of Pto kinase, consistent with our previous prediction of a peptide or molecule suppressing kinase activity.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of Pto Mutants in N. benthamiana and Escherichia coli**—All constructs were cloned into pCRII (Invitrogen) and sequenced to exclude PCR errors. For expression in planta, constructs were cloned in the pTFS40 binary vector (26). Recombinant binary vectors were transferred to Agrobacterium tumefaciens strain GV2260 for expression in planta, and the presence of the vector was confirmed by screening DH5α or BL21-CodonPlus (Stratagene, La Jolla, CA). Transformed bacterial cultures were grown in SOB media supplemented with appropriate antibiotics to \( A_{600} = 0.4 \) at 37 °C, and expression was induced with isopropyl \( β\)-D-1-thiogalactopyranoside at 16 °C overnight. Alternatively, Pt was expressed with a C-terminal His6 epitope tag with the pBAD expression system (Invitrogen). Expression of recombinant Pto-His6 was induced with l-arabinose at 16 °C overnight as for the GST fusion proteins.

**Recombinant Protein Purification—GST fusion proteins were purified over glutathione-Sepharose columns (GE Healthcare) as described (16, 25).** GST-pto1K66N was used to be the substrate in Pto kinase assays was expressed as described (26) and purified over GSTrap FF columns as for Pto. Where required, GST-pto1K66N was treated with thrombin protease (GE Healthcare) to cleave the GST moiety (25).

For Pto-His6 purification, cells from induced cultures were pelleted, resuspended in 25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 20 mM imidazole, and bacterial protease inhibitor mixture (Sigma), lysed by sonication, and centrifuged at 13,000 \( \times \) g for 30 min. Crude extracts were filtered through 0.45-μm filters and applied to HisTrap FF columns (GE Healthcare). Bound proteins were eluted with 25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 500 mM imidazole, and finally desalted through PD-10 columns (GE Healthcare) against 25 mM Tris-HCl (pH 7.5).

**Protein Immunoprecipitation Using Epitope Tags—FLAG epitope-tagged proteins transiently expressed in N. benthamiana leaves were immunoprecipitated from crude extracts with anti-FLAG® M2 affinity gel (Sigma) as described (16).** Immune complexes were collected by centrifugation, washed with 1× Tris-buffered saline (TBS), resuspended in 1× TBS, 50% (v/v) glycerol, and stored at −20 °C for subsequent kinase assays.

**Peptide Synthesis**—Peptides corresponding to Pto N-terminal residues 2–10 (Gly-Ser-Lys-Tyr-Ser-Lys-Ala-Thr-Asn) were synthesized commercially (Sigma Genosys). These were produced in the myristoylated (at Pto Gly-2; myr-Pto2-10) and nonmyristoylated (Pto2-10) forms. The protein kinase Ca pseudosubstrate inhibitor peptides myr-PKC, and PKC (Leu-His-Gln-Arg-Arg-Gly-Ala-Ile-Leu-Lys-Gln-Ala-Lys-Val-His-His-Val-Lys-Cys-NH2, myr-PKC, non myristoylated, PKC, were obtained commercially (Calbiochem). The hemagglutinin (HA) peptide (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) was from Roche Applied Science.

**MAPK Activation, Immunoprecipitation, and Western Blotting—Arabidopsis thaliana** ecotype Landsberg erecta cell suspension cultures were treated at room (ambient) temperature for 10 min with 100 μM flagellin peptide (fli22) (27). Cells were collected by vacuum filtration, ground under liquid nitrogen to a fine powder, and suspended in 100 mM Tris-HCl (pH 7.5), 50% (v/v) glycerol, 50 mM sodium pyrophosphate, 1 mM sodium molybdate, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 25 μM β-glycerophosphate, 15 mM EDTA, 5 mM EGTA, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonyl fluoride, protein protease inhibitors mixture (Sigma). Following centrifugation at 13,000 \( \times \) g for 30 min at 4 °C, crude extracts were filtered through 0.45-μm filters. Activated AtMAPK3 and AtMAPK6 were immunoprecipitated with specific antisera, and immune complexes were precipitated with protein A-Sepharose (GE Healthcare), washed with the same buffer,
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and finally resuspended in kinase buffer (see under “In Vitro Kinase Activity Assays”).

Activated AtMAPK3 and AtMAPK6 were detected by Western blotting using α-DP-ERK (Sigma) as the primary antibody. This reacts specifically with the epitope containing the phosphorylatable Thr and Tyr residues within the regulatory site of active MAPK. Immunodetection was performed with an ECL chemiluminescence reagent (GE Healthcare).

In Vitro Kinase Activity Assays—Pto in vitro kinase assays contained the following in a total volume of 50 μl: 1 μg of recombinant Pto or 10 μl of immunoprecipitated Pto-FLAG (1:1 (v/v) suspension in 1× TBS, 50% (v/v) glycerol), 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM MnCl₂, 1 mM dithiothreitol, 20 μM ATP, 183 kBq of [γ-32P]ATP (PerkinElmer Life Sciences). For assays of Pto trans-phosphorylation activity, either full-length GST-pto[N-10] or cleaved pti1K⁹⁶⁶N (1 μg) was also included in kinase reactions as required. Reactions were initiated by addition of the substrate, incubated at 25 °C for 7.5 min, and terminated by addition of SDS-PAGE loading buffer and boiling for 5 min. Under these assay conditions, incorporation of radiolabel was found to be linear with time and the enzyme concentration used. To assess the effect of inhibitors on Pto kinase activity, each inhibitor was included in the kinase assays at the concentrations indicated under “Results.” At the end of each assay, samples were loaded on SDS-PAGE. Post-electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes, which were either stained with Coomassie Brilliant Blue R-250 to confirm equivalent loading or used for Western blots with specific antisera against Pto (25).

In vitro kinase activity of GST-Fen and GST-Pti1 kinases was assayed as described with the QuantityOne software (Bio-Rad) after specific incorporation (PhosphorImager signal) and amount of protein measured. Pto trans-phosphorylation activity was reduced by more than 90% at myr-Pto2–10 concentrations higher than 100 μM. The half-maximal inhibitory concentration (IC₅₀) was estimated to be ~50 μM. Conversely, neither Pto2–10 nor the unrelated hemagglutinin peptide (HA) had a significant effect on Pto kinase activity. Pto autophosphorylation activity was also suppressed by myr-Pto2–10, albeit to a lesser extent than trans-phosphorylation (Fig. 1b). We also tested whether Pto expressed in planta was inhibited by myr-Pto2–10-pto[N-10] fused to a sequence encoding a C-terminal FLAG epitope was transiently expressed in N. benthamiana leaves under control of the cauliflower mosaic virus 35S promoter. The protein was immunoprecipitated from crude extracts with anti-FLAG® M2-agarose beads and used for in vitro kinase assays in the presence of myr-Pto2–10 and Pto2–10. As for GST-pto[N-10], severe inhibition of kinase activity was effected at increasing concentration of myr-Pto2–10 but not Pto2–10 (Fig. 1c).

We next asked whether the inhibition caused by myr-Pto2–10 could be observed in the presence of a myristoylated peptide of random sequence. To test this, we used two peptides unrelated to Ptoα–10, myr-PKCα and PKCα differing only for myristoylation status. These peptides function as pseudosubstrate inhibitors of protein kinase C, isoform θ (PKCθ) activity (29). A progressive reduction of both the trans- (Fig. 1d) and auto-phosphorylation (Fig. 1e) activities of GST-pto[N-10] was observed in the presence of increasing concentrations of the myristoylated peptide (myr-PKCθ).

RESULTS

The in Vitro Kinase Activity of Pto Is Inhibited in Trans by the Myristoylated N-peptide—We previously identified the Pto NRP, a hydrophobic surface on Pto overlapping the kinase catalytic cleft that negatively regulates signaling (25). We hypothesized that this patch is normally occupied by an unknown molecule acting to repress kinase activity. This could be part of Pto itself or derived from another molecule. Removal of the negative regulator was predicted to be sufficient to activate downstream signaling. We found that N-myristoylation was important for the activity of CGF forms of Pto, downstream of kinase activity (16). Given its important functions and location outside of the kinase domain, we investigated whether the myristoylated N terminus of Pto is a candidate for the proposed regulatory molecule. To test this, we explored the ability of a peptide comprising amino acids 2–10 to repress Pto kinase activity in vitro.

We expressed pto[N-10] in E. coli as a C-terminal fusion with the GST protein. This protein lacks 10 N-terminal amino acids and was shown previously to be an active kinase in vitro (16). To test the ability of residues 2–10 (not including Met-1, which is cleaved after translation) to repress Pto kinase activity, we synthesized the unmyristoylated (Pto2–10) and myristoylated (myr-Pto2–10) modified on Gly-2) forms of this peptide. Both were included in in vitro kinase activity assays of GST-pto[N-10] with pti1K⁹⁶⁶N as the substrate. This assay allows measurement of both the auto- and trans-phosphorylation activities of Pto. Increasing the concentration of the myr-Pto2–10 peptide effected significant inhibition of the trans-phosphorylation activity of GST-pto[N-10] against pti1K⁹⁶⁶N (Fig. 1a). Pto trans-phosphorylation activity was reduced by more than 90% at myr-Pto2–10 concentrations higher than 100 μM. The half-maximal inhibitory concentration (IC₅₀) was estimated to be ~50 μM. Conversely, neither Pto2–10 nor the unrelated hemagglutinin peptide (HA) had a significant effect on Pto kinase activity. Pto autophosphorylation activity was also suppressed by myr-Pto2–10, albeit to a lesser extent than trans-phosphorylation (Fig. 1b). We also tested whether Pto expressed in planta was inhibited by myr-Pto2–10-pto[N-10] fused to a sequence encoding a C-terminal FLAG epitope was transiently expressed in N. benthamiana leaves under control of the cauliflower mosaic virus 35S promoter. The protein was immunoprecipitated from crude extracts with anti-FLAG® M2-agarose beads and used for in vitro kinase assays in the presence of myr-Pto2–10 and Pto2–10. As for GST-pto[N-10], severe inhibition of kinase activity was effected at increasing concentration of myr-Pto2–10 but not Pto2–10 (Fig. 1c).

We next asked whether the inhibition caused by myr-Pto2–10 could be observed in the presence of a myristoylated peptide of random sequence. To test this, we used two peptides unrelated to Ptoα–10, myr-PKCα and PKCα differing only for myristoylation status. These peptides function as pseudosubstrate inhibitors of protein kinase C, isoform θ (PKCθ) activity (29). A progressive reduction of both the trans- (Fig. 1d) and auto-phosphorylation (Fig. 1e) activities of GST-pto[N-10] was observed in the presence of increasing concentrations of the myristoylated peptide (myr-PKCθ).

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The rate and the extent of inhibition caused by myr-PKC\textsubscript{\beta} were comparable with that effected by myr-Pto\textsubscript{2-10}. The estimated IC\textsubscript{50} for the inhibition of GST-pti\textsubscript{[N-10]} trans-phosphorylation activity by myr-PKC\textsubscript{\beta} was \textasciitilde55 \textmu M, similar to myr-Pto\textsubscript{2-10} (\textasciitilde50 \textmu M). Comparable with Pto\textsubscript{2-10}, the nonmyristoylated PKC\textsubscript{\beta} peptide did not suppress Pto kinase activity. Therefore, we conclude that Pto residues 2–10 do not contribute to suppression of Pto kinase activity in vitro.

Free Myristate Inhibits Pto Kinase Activity in Vitro—The results above suggest that inhibition of Pto kinase activity by myr-Pto\textsubscript{2-10} was because of the myristate moiety on Gly-2. To test this, we added myristate directly to kinase assays in the absence of conjugated peptide. These experiments used myristate-coenzyme A (myr-CoA) as the inhibitor to overcome the insolubility of myristate in aqueous solution. Severe inhibition of both the trans- and auto-phosphorylation activities of recombinant Pto was observed in the presence of myr-CoA (Fig. 2). Conversely, kinase activity was not significantly inhibited by CoA alone; therefore, inhibition was because of the myristate moiety. Less than 10% of kinase activity was retained at myr-CoA levels higher than 20 \textmu M. The corresponding IC\textsubscript{50} for inhibition of trans-phosphorylation activity by myr-CoA was \textasciitilde15 \textmu M, substantially lower than the IC\textsubscript{50} of myr-Pto\textsubscript{2-10} (\textasciitilde50 \textmu M). Similar results were obtained with Pto-FLAG purified from \textit{N. benthamiana} leaf extracts (data not shown).

We reasoned that the inhibition of kinase activity by myristate might be attributable to its hydrophobic nature. In this case, other fatty acids might cause similar repression of Pto kinase activity. Therefore, we substituted myr-CoA in the kinase assays with CoA derivatives of both saturated (lauric acid, C12:0; palmitic acid, C16:0) and unsaturated (oleic acid, C18:1; linoleic acids, C18:2) fatty acids. Severe inhibition of Pto kinase activity was effected by all fatty acids tested (Fig. 3). The extent of suppression in all treatments was similar to that caused by myristate. These results indicate that the hydrophobic nature of the inhibitor is important for suppression of kinase activity in vitro.

Inhibition of Kinase Activity by N-Myristoylation Declines with Evolutionary Distance from Pto—The protein kinase family is monophyletic, and all crystallized eukaryotic protein kinases possess a common fold (30). Therefore, we were interested to test the generality of kinase activity repression by myr-Pto\textsubscript{2-10} or myristate. If the observed inhibition is specific to Pto, then less closely related protein kinases should not be affected to the same extent by either inhibitor. We first investigated the in vitro activity of two mitogen-activated protein kinases (MAPK) from \textit{A. thaliana}, AtMAPK3 and AtMAPK6. These enzymes are stimulated by a
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We next investigated the effect of myr-CoA on protein kinases more closely related to Pto. Fen is a Pto family member with ~80% identity to Pto, is capable of Prf-dependent signaling (13), and its N terminus is an efficient substrate for A. thaliana myristoyl-CoA:protein N-myristoyltransferase (33). Pti1 interacts with Pto in yeast two-hybrid assays and may participate in defense-related signaling in planta (7). Pti1 shares ~39% amino acid identity with Pto within the kinase domain (12). Fen and Pti1 were expressed in E. coli as C-terminal GST fusions, and their autophosphorylation potentials were assessed in vitro in the presence of myr-CoA. Similar to Pto, increasing the concentration of myr-CoA affected significant suppression of both GST-Fen and GST-Pti1 autophosphorylation capabilities (Fig. 4b). GST-Fen and GST-Pti1 activities were suppressed by ~40% at myr-CoA levels corresponding to the IC50 of Pto inhibition. The estimated IC50 values were ~25 μM (GST-Fen) and 22 μM (GST-Pti1) (Table 1). We also examined the effect of myr-CoA on the autophosphorylation activity of the kinase domain of SYMRK of Lotus japonicus, which shares 37% identity with Pto (28). Recombinant SYMRK kinase domain (fused to an N-terminal His6 epitope tag; His6-SYMRK) activity against MBP was moderately suppressed by myr-CoA. Approximately 25% of activity was retained at myristate levels that resulted in almost complete inhibition of Pto activity (Fig. 4).

Taken together, the data indicate that inhibition of kinase activity by myristate declines with evolutionary distance from Pto.

In Vivo Myristoylation on Gly-2 Reduces Pto Kinase Activity—Because exogenous myristate was found to strongly inhibit Pto kinase activity, it follows that removal of the fatty acid from the native protein should de-repress enzymic activity. Pto is myristoylated on the glycine residue at position 2, and mutation of Gly-2 to Ala abolishes myristoylation in vivo (16). Hence, we expressed Pto-FLAG and ptoG2A-FLAG transiently in N. benthamiana leaves under control of the cauliflower mosaic virus 35S promoter. Recombinant proteins were purified from crude extracts by immunoprecipitation with anti-FLAG® M2-agarose beads, and both auto- and trans-phosphorylation capabilities were assessed as before. Elimination of in vivo myristoylation in the ptoG2A mutant resulted in de-repression of kinase activity in vitro. The nonmyristoylated protein exhibited ~30 and 40% higher auto- and trans-phosphorylation activity, respectively, relative to the myristoylated wild type Pto (Fig. 5a).

We then investigated the kinetic properties of the Pto and ptoG2A proteins purified from plant extracts. Substrate saturation curves were prepared for both Pto-FLAG and ptoG2A-FLAG (Fig. 5b). Pto activity increased with increasing concentration of GST-pto1K96N, and ptoG2A-FLAG activity was consistently higher than that of Pto-FLAG. The initial data were transformed to double-reciprocal plots for estimation of

![Figure 2](image2.png)

**Figure 2.** *In vitro* kinase activity of Pto is inhibited by myristate. Effect of myristate on trans-phosphorylation (a) and autophosphorylation activity of Pto (b). In vitro activity of recombinant GST-Pto was assayed in the presence of increasing concentration of myr-CoA or CoA. Results are expressed as % of the activity in the absence of the inhibitor and are averages from at least three independent experiments performed in duplicate ± S.E. (error bars). (CoA), □, myr-CoA; □, CoA.

![Figure 3](image3.png)

**Figure 3.** Effect of saturated and unsaturated fatty acids on GST-pto[N-10] kinase activity *in vitro*. Fatty acids (as the corresponding CoA esters) or CoA (control) was added at increasing concentrations to GST-pto[N-10] kinase assays with pti1K96N as the protein substrate. Autoradiographs are shown for detection of radiolabeled species. The position of migration of GST fusion proteins and pti1K96N is indicated. Equivalent loading was verified by Coomassie stain of the autoradiograph gels (not shown).

### Table 1

| Identity to Pto | Overall Kinase domain | Auto-
|                | IC50 (μM) | Trans-
|                | %       | phosphorylation | phosphorylation |
| AtMAPK3        | 14      | ND*a          | ND         | 20        |
| Pti1           | 35      | 39            | 22         | NA*b      | 85        |
| SYMRK          | 37      | 44            | 45         | 40        | 75        |
| Fen            | 78      | 79            | 25         | NA        | >95       |
| Pto            | 15      | 15            | 15         | >95       |

*a ND indicates not determined because the IC50 was not achieved within the concentration range used.

*b NA indicates not applicable.

A wide array of abiotic stresses and biotic elicitors, including the peptide flg22 derived from bacterial flagellin (27, 31, 32), and share little identity to Pto (Table 1). AtMAPK3 and AtMAPK6 were immunoprecipitated from flg22-treated A. thaliana cell suspension cultures and the immune complexes used directly in *in vitro* kinase assays with Phos32 protein as the substrate. Inclusion of either myr-Pto2–10 (not shown) or myr-CoA in the kinase assays did not have a pronounced effect on the activity of AtMAPK3. At myr-CoA levels that caused more than 90% inhibition of Pto kinase, AtMAPK3 enzymic activity was repressed only by ~25% (Fig. 4). Results similar to the AtMAPK3 data were also obtained with AtMAPK6 (data not shown).
apparent kinetic parameters (Fig. 5c). The $K_{m}$ value estimated for ptoG2A-FLAG was $\sim 1.9 \mu M$ and is of the same order of magnitude as that estimated by Sessa et al. (34) for the similarly nonmyristoylated GST-Pto (4.1 $\mu M$). However, it is significantly lower than the $K_{m}$ estimate for the myristoylated wild type Pto-FLAG ($\sim 13 \mu M$). Together, the data suggest that elimination of the myristoylation potential of Pto results in higher activity in the absence of the inhibitor and are averages of at least two independent experiments. This is consistent with occupation of the catalytic cleft by the myristoylated N terminus of Pto.

The Myristoylated N Terminus of Pto Suppresses Kinase Activity through the Negative Regulatory Patch—In previous work we hypothesized that Pto signaling is suppressed by a molecule or peptide that occupies the NRP that overlaps the catalytic cleft of Pto (25). Mutations within the NRP confer catalytic cleft of Pto (25). Mutations within the NRP confer catalytic potential of Pto without loss of kinase activity (25). We assessed the extent of interaction with the NRP, we expressed GST-pto[N-10]$^{I214D}$ in E. coli as described for GST-pto[N-10]. Purified GST-pto[N-10]$^{I214D}$ exhibited both auto- and trans-phosphorylation activities in vitro as reported previously for the full-length GST-Pto fusions (25) (Fig. 6a). Inclusion of myr-Pto$_{2-10}$ but not Pto$_{2-10}$ in the kinase assays resulted in moderate inhibition of GST-pto[N-10]$^{I214D}$ activity (Fig. 6b). Thus, trans-phosphorylation activity. GST-pto[N-10]$^{I214D}$ was suppressed to a lesser extent than GST-pto[N-10]. At the maximum inhibitor concentration, almost 40% of kinase activity was retained by the GST-pto[N-10]$^{I214D}$ protein. This is significantly higher than the activity of GST-pto[N-10] which, at the same inhibitor concentration, exhibited less than 10% activity. These differences were also reflected in both the rate of inhibition and the estimated $IC_{50}$ values (GST-pto[N-10] $\sim 50 \mu M$ and GST-pto[N-10]$^{I214D}$ $\sim 100 \mu M$). No significant differences between GST-pto[N-10] and GST-pto[N-10]$^{I214D}$ were evident in either the rate or the extent of inhibition of autophosphorylation activity by myr-Pto$_{2-10}$ (cf. Fig. 1b and data not shown). Thus, Ile-214 is required for inhibition of Pto trans-phosphorylation activity by myr-Pto$_{2-10}$.

Data above show that the repressive effect of myr-Pto$_{2-10}$ is because of the myristate moiety. To investigate the effect of myristate on pto$^{I214D}$ activity, we included myr-CoA in in vitro kinase assays of full-length Pto and pto$^{I214D}$ proteins. These were expressed in E. coli fused to C-terminal His$_6$ tags (Pto-His$_6$ and pto$^{I214D}$-His$_6$ respectively) and purified by IMAC. Recombinant pto$^{I214D}$-His$_6$ was an active kinase comparable with Pto-His$_6$ (Fig. 6c) (25). We assessed the in vitro kinase capabilities of these two proteins, together with GST-pto[N-10] and GST-pto[N-10]$^{I214D}$, myr-CoA severely inhibited the kinase activities of pto$^{I214D}$-His$_6$ and GST-pto[N-10]$^{I214D}$. The extent of inhibition of the I214D forms was similar to that of Pto-His$_6$ and GST-pto[N-10], respectively (Fig. 6c). We estimated the $IC_{50}$ values for inhibition of trans-phosphorylation activ-

![Figure 4](image)

**FIGURE 4. Inhibition of kinase activity by myr-CoA declines with evolutionary distance from Pto.** a, effect of myr-CoA on the *in vitro* autophosphorylation activity of AtMAPK3 ( ), His$_6$SYMRK ( ), GST-Pti1 ( ), GST-Fen ( ) and GST-Pto ( ). b, effect of myr-CoA on *in vitro* trans-phosphorylation activity of AtMAPK3 ( ) against C Phos32, His$_6$SYMRK ( ) against MBP, and GST-Pto ( ) against pti$^{I214D}$. Results are expressed as % of the activity in the absence of the inhibitor and are averages of at least two independent experiments + S.E. (error bars). Similar results were obtained in the presence of myr-Pto$_{2-10}$ (data not shown).

![Figure 5](image)

**FIGURE 5. Elimination of the *in vivo* myristoylation potential of Pto de-represses kinase activity.** a, wild type (WT) Pto-FLAG (closed bars) or pto$^{G2A}$-FLAG (open bars) were expressed transiently in *N. benthamiana* leaves, immunoprecipitated, and used in *in vitro* kinase assays with GST-pto$^{I214D}$ as the substrate. Kinase activity was expressed as % of that exhibited by wild type Pto and are average values from three independent experiments, each performed in triplicate + S.E. (error bars). b, GST-pto$^{I214D}$ saturation curves for wild type Pto-FLAG ( ) and pto$^{G2A}$-FLAG ( ) transiently expressed and purified from *N. benthamiana* leaves. Results are averages + S.E. (error bars). c, the data in b were transformed to double-reciprocal plots to estimate the kinetic parameters of myristoylated and nonmyristoylated Pto forms. ( ), Pto-FLAG; ( ), pto$^{G2A}$-FLAG.
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**DISCUSSION**

Protein kinases are potent signaling molecules whose activity is under acute control to prevent aberrant signaling. Although the structures of protein kinases in the active conformation are highly similar, inhibitory mechanisms tend to be specific to individual kinases (30). The tomato Pto kinase expressed either in bacteria or in plants possesses intrinsic enzymic activity in vitro so is not misfolded; yet its signaling in vivo requires regulated kinase capability. Therefore, Pto enzymic activity must be repressed in vivo by a mechanism that has not previously been detectable in in vitro experiments. Moreover, signaling by Pto in the active conformation is independent of kinase activity (25). Thus, activated Pto must be distinguished from the inactive form by a change in conformation, rather than by enhanced kinase activity. We hypothesized that the Pto NRP is normally occupied by a regulatory molecule that represses kinase-dependent activation and is released by ligand-dependent kinase activity (25). Here we show that the myristoylated Pto N terminus is a candidate for this molecule. N-Myristoylation is a co-translational modification specific to eukaryotes (15), so recombinant Pto protein produced in E. coli is not acylated. We show that myristate is a repressor of Pto kinase activity, either supplied in trans as free myristate or as an acylated peptide or in cis as part of the native molecule expressed in planta. Furthermore, the importance of N-myristoylation for signaling of CGF forms of Pto (16), downstream of the requirement for kinase activity, demonstrates that acylation also plays a positive regulatory role in Pto signaling. Thus, the myristoylated N terminus of Pto fits the previously proposed model, in which expulsion of a negative regulatory molecule from the NRP is important for signaling.

Several lines of evidence demonstrate that myristate is a suppressor of Pto kinase activity. We showed that the myr-Pto2-10 peptide is a potent inhibitor of Pto trans-phosphorylation. Surprisingly, the N-peptide itself was incapable of kinase inhibition. Thus, Pto residues 2–10 do not contribute to suppression of kinase activity in vitro. This is different from pseudosubstrate inhibition observed in a number of protein kinases, such as the protein kinase C (PKC) (29) and the myosin light chain kinase (35). Our data further indicate that there is no sequence requirement for the inhibitory activity of the myristoylated peptide, because both myr-Pto2-10 and myr-PKCα suppressed Pto kinase activity to the same extent. In contrast, an acylated PKC pseudosubstrate peptide, but not a similarly acylated unrelated peptide, was an efficient and selective inhibitor of PKC activity (9).

The inhibitory activity of myr-Pto2-10 can be ascribed to the fatty acid moiety, because free myristate delivered as myr-CoA severely suppressed Pto kinase capability. Importantly, acylated Pto expressed in planta was less active than the myristoylation-deficient G2A mutant. Deletion of Pto acylation capability was associated with enhanced kinase activity and a significant increase in substrate specificity. Similarly, myristoylation-deficient forms of the nonreceptor tyrosine kinase c-Abl and AMP-activated protein kinase were more active compared with the acylated wild type forms (22, 23). We suggest that the repressive effect of myris-
Regulation of Pto Kinase Activity by N-Myristoylation

Several lines of evidence suggest that the myristoylated N terminus lies within or close to the catalytic cleft of Pto. We showed previously that pto[N-10] was a less active kinase than wild type Pto, implying a role for residues 2–10 in kinase activity (16). Similarly, N-acylation decreased substrate affinity and enzyme activity (Fig. 5). Residues 11–20 of Pto were required for optimal interaction with AvrPto (but not AvrPtoB) in yeast (16). This is striking because the main site of AvrPto interaction overlaps the catalytic cleft (25). The NRP residue Ile-214 provides an important point of intersection between regulatory mechanisms of Pto in vivo and in vitro. Acidic substitution of this residue results in the CGF phenotype in vivo, interpreted previously as loss of negative regulation due to interruption of an inhibitory intermolecular interaction. Conversely, the same mutation reduces the inhibitory effect of myr-Pto2–10. Synthesis of these observations allows a model in which Ile-214 is a point of attachment with the inhibitory N terminus. In the absence of the Avr proteins, we suggest that the myristoylated Pto N terminus occupies the NRP and represses kinase activity in concert with the Prf protein (Fig. 7). Myristate attachment at Gly-2 allows docking of the N terminus onto the hydrophobic surface of the patch. The Avr proteins are delivered into the host cell, where they interact with the Pto-Prf complex, targeting the same region on Pto that is normally occupied by the myristoylated N terminus. The negative regulator is displaced from the NRP, allowing de-repression of kinase activity, regulatory phosphorylation, and subsequent Pto activation.

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