

Bypassing Kinase Activity of the Tomato Pto Resistance Protein with Small Molecule Ligands*[§]

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The tomato (*Solanum lycopersicum*) protein kinase Pto confers resistance to *Pseudomonas syringae* pv. *tomato* bacteria expressing the AvrPto and AvrPtoB effector proteins. Pto specifically recognizes both effectors by direct physical interactions triggering activation of immune responses. Here, we used a chemical-genetic approach to sensitize Pto to analogs of PP1, an ATP-competitive small molecule inhibitor. By using PP1 analogs in combination with the sensitized Pto (Pto^{as}), we examined the role of Pto kinase activity in effector recognition and signal transduction. Strikingly, while PP1 analogs efficiently inhibited kinase activity of Pto^{as} *in vitro*, they enhanced interactions of Pto^{as} with AvrPto and AvrPtoB in a yeast two-hybrid system. In addition, in the presence of PP1 analogs, Pto^{as} bypassed mutations either at an autophosphorylation site critical for the Pto-AvrPto interaction or at catalytically essential residues and interacted with both effectors. Moreover, in the presence of the PP1 analog 3MB-PP1, a kinase-deficient form of Pto^{as} triggered an AvrPto-dependent hypersensitive response *in planta*. These findings suggest that, rather than phosphorylation *per se*, a conformational change likely triggered by autophosphorylation in Pto and mimicked by ligand binding in Pto^{as} is a prerequisite for recognition of bacterial effectors. Following recognition, kinase activity appears to be dispensable for Pto signaling *in planta*. The chemical-genetic strategy applied here to develop specific small molecule inhibitors of Pto represents an invaluable tool for the study of biological functions of other plant protein kinases *in vivo*.

Protein kinases regulate a wide variety of cellular processes and signal transduction pathways in plants. The large number of protein kinases present in plant proteomes (>1,000 in *Arabidopsis*, >1,500 in rice) makes it a very difficult challenge to assign precise roles to each individual kinase (1, 2). To study protein kinase functions, strategies based on single knock-out mutations and gene silencing are widely employed. However, such efforts are often compromised by lethality, in the case of essential genes, or by functional redundancy and cellular homeostasis. Cell-permeable inhibitors that are highly specific for

individual protein kinases would allow overcoming the limitations of genetic approaches to enzyme inactivation and thus represent invaluable tools for assisting in the definition of plant kinase functions. However, while small molecule inhibitors have been recently developed in great numbers for animal protein kinases, their use in the study of plant kinases is still very limited.

Specific kinase inhibitors would be particularly useful for elucidating modes of activation and signaling of protein kinases that serve as receptors in plant innate immunity. Two major classes of receptors detect pathogen invasion and activate immunity in plants (3, 4). One class consists of pattern recognition receptors that are located on the plant cell surface and detect pathogen-associated molecular patterns, which are conserved in many microbes (5). Among the few pattern recognition receptors that have been isolated to date, the receptors of bacterial flagellin and elongation factor Tu encode transmembrane proteins with a cytoplasmic kinase domain (6, 7). The second class of receptors involved in plant innate immunity is represented by resistance (R)³ proteins that specifically recognize virulence proteins, which are commonly referred to as pathogen effectors (8). Kinase catalytic domains are found in certain R proteins either without any additional functional domain (*e.g.* tomato Pto) or in combination with extracellular leucine-rich repeats and transmembrane domains (*e.g.* rice Xa21 and barley Rpg1 and Rpg5) (9, 10).

The tomato (*Solanum lycopersicum*) R protein Pto encodes a serine/threonine protein kinase and confers immunity to the Gram-negative bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) expressing either AvrPto or AvrPtoB (11). During pathogenesis, AvrPto and AvrPtoB are among the ~30 *Pst* effector proteins that are delivered into the host cell by the bacterial type III secretion system to promote disease and interfere with host immunity (12–17). Pto specifically recognizes the presence of AvrPto and AvrPtoB in the plant cell by direct physical interactions that result in the activation of the hypersensitive response (HR), an array of defense responses including cell death at the site of pathogen infection (11, 18). Pto-mediated immunity requires the nucleotide binding site/leucine-rich repeats (NBS-LRR) protein Prf that associates with Pto in a high molecular weight complex (19). Functional screens based on

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³ The abbreviations used are: R, resistance; CaMV, *Cauliflower mosaic virus*; GST, glutathione S-transferase; HR, hypersensitive response; MBP, maltose-binding protein; NBS-LRR, nucleotide binding site/leucine-rich repeats; PP1, 1-*tert*-butyl-3-*p*-tolyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; *Pst*, *Pseudomonas syringae* pv. *tomato*; Pto^{as}, analog-sensitive Pto; RG, Rio Grande.

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virus-induced gene silencing identified MAP kinase cascades and additional signaling proteins acting downstream of the Pto/Prf complex (20, 21).

Extensive biochemical studies revealed the ability of Pto to autophosphorylate at multiple sites and to phosphorylate substrate proteins *in vitro* (22, 23). However, the role of Pto kinase activity in recognition of bacterial effectors and signal transduction has been controversial for a long time and not yet entirely resolved. Early studies examined the requirement of Pto kinase activity for effector recognition by analyzing the effect of point mutations at conserved catalytic residues or autophosphorylation sites on the Pto-AvrPto or Pto-AvrPtoB physical interaction in a yeast two-hybrid system (23–26). In most instances kinase-deficient forms of Pto did not interact with AvrPto or AvrPtoB, suggesting a requirement of Pto kinase activity for effector recognition; however, a few exceptions supported opposite conclusions (25, 26). More recently, structural and *in vitro* biochemical analysis suggested that stabilization of the Pto P+1 loop by phosphorylation at Thr-199 is a prerequisite for AvrPto recognition (27). As for the role of Pto kinase activity in signal transduction, evidence that kinase activity is dispensable after effector recognition derived from constitutive gain-of-function Pto mutants that elicit HR in an effector-independent manner, while not displaying kinase activity *in vitro* (25, 26). In contrast, Pto kinase activity appears to be required for signaling based on a mutation at the Pto autophosphorylation site Ser-198, which is required for the elicitation of the HR but dispensable for the interaction with AvrPto (23).

To elucidate further the role of Pto kinase activity in recognition of AvrPto and AvrPtoB, and in effector-dependent activation of immune responses, we used a chemical-genetic approach that renders a given kinase uniquely susceptible to analogs of 1-*tert*-butyl-3-*p*-tolyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine (PP1), an ATP-competitive small molecule inhibitor (28, 29). In this approach, substitution of a conserved and bulky residue, termed the *gatekeeper* residue, with alanine or glycine generates a unique pocket in the ATP binding site of the kinase of interest, which is not present in any wild-type kinase. The sensitized mutant can then be selectively and potently inhibited by ATP-competitive small molecules that contain substituents occupying the novel pocket. This strategy has been successfully applied to specifically inhibit catalytic activity of protein kinases from multiple organisms including plants, yeast, and mammalian cell cultures by using PP1 analogs (29–33).

We previously engineered the Pto ATP-binding site to contain a unique enlarged binding pocket while retaining catalytic activity (34). In the analog-sensitive Pto allele (Pto^{as}) the *gatekeeper* residue was mutated to alanine (Y114A) and a second site mutation (L68I) was introduced to rescue the severely reduced kinase activity of Pto(Y114A) (34). In the present study, we confirmed that Pto^{as} displays functional properties of wild-type Pto and is specifically targeted by PP1-derived small-molecule inhibitors. Binding of PP1 analogs to the enlarged ATP-binding site of Pto^{as} resulted in specific inhibition of kinase activity *in vitro*, yet surprisingly allowed kinase-deficient forms of Pto^{as} to bind bacterial effectors in yeast and to elicit an

effector-dependent HR *in planta*. These results, interpreted in combination with structural data (27), suggest that a conformational change, possibly elicited by autophosphorylation in wild-type Pto and mimicked by binding of PP1 analogs in Pto^{as}, enables Pto to recognize bacterial effectors. In addition, our findings support the notion that kinase activity is dispensable for Pto-mediated signal transduction. Finally, the chemical-genetic strategy used here for Pto may be applied to the *in vivo* study of other plant protein kinases.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutagenesis of Pto was performed in the shuttle vector pTEX containing the *Pto* coding region under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (35), or in the plasmids pGEX-4T1 and pEG202, containing the *Pto* coding region fused to glutathione *S*-transferase (GST) and to the LexA DNA binding domain, respectively (22, 23). Site-specific mutations were introduced using the QuikChange kit (Stratagene). Sequences of oligonucleotides used for mutagenesis are listed in supplemental Table S1. The insertion in the gene of the desired mutations was confirmed by sequencing.

Protein Expression in *Escherichia coli*—Constructs for the expression in *E. coli* of MPK2 as a maltose-binding protein (MBP) fusion and of Pto, Pto^{as}, Pti1, and the kinase-deficient mutant Pti1(K96N) as GST fusions were described previously (22, 34, 36, 37). GST fusions were expressed in the *E. coli* strain DH12S grown to $A_{600} = 1$. Expression of recombinant proteins was induced by 0.05 mM isopropyl β -D-thiogalactopyranoside for 6 h at 24 °C. GST fusion proteins were then affinity-purified by using glutathione-agarose beads (Sigma). Expression in *E. coli* and purification of MBP fusions with amylose resin were performed according to the instructions of the manufacturer (New England Biolabs).

Kinase Activity Assays—Kinase assays to test autophosphorylation of Pto forms, Pti1, and MPK2 were performed *in vitro* with 2 μ g of the recombinant kinase in 20 μ l of kinase reaction buffer (50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 10 mM MnCl₂, and 20 mM ATP) containing 1 μ Ci of [γ -³²P]ATP (3,000 Ci/mmol; Amersham Biosciences). When testing phosphorylation of Pti1(K69N) by Pto forms, 2 μ g of GST-Pti1(K69N) was included in the reaction mixture. PP1 analogs were synthesized as described (38, 39) and their effect on kinase activity was tested at the final concentration of 1 μ M. As a control, assays were performed in the absence of inhibitors with an equivalent volume of DMSO. Reactions were incubated for 10 min at room temperature and stopped by the addition of 10 mM EDTA. At this time, phosphate incorporation was found to be linear for the amount of enzyme used in the reaction. Proteins were then fractionated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), stained by Coomassie Brilliant Blue R-250, and the dried gel was analyzed by Phosphorimager (Fujifilm FLA-2000) or exposed to autoradiography. IC₅₀ values for the PP1 analogs 3MB-PP1, 1NM-PP1, 2Na-PP1, and Ac-PP1 were determined by measuring the counts per minute of ³²P transferred by GST-Pto^{as} to GST-Pti1(K96N). Various inhibitor concentrations ranging from 1 nM to 1 μ M were added to kinase reactions performed as described above. IC₅₀ values were cal-

culated as the concentration of inhibitor at which ^{32}P incorporation into GST-Pti1(K96N) was 50% of that in control reactions carried out in the absence of inhibitor.

Yeast Two-hybrid Assays—Yeast two-hybrid interactions were tested in the yeast strain EGY48 using standard protocols (40). Yeast were transformed sequentially with the lacZ reporter plasmid pSH18–34, the bait plasmid pEG202 expressing LexA DNA-binding domain fused to wild-type or mutant Pto, and the prey plasmid pJG4–5 expressing the GAL4 activation domain fused either to AvrPto, AvrPtoB_{1–387} or their mutant forms. The Pto bait vector as well as the AvrPto and AvrPtoB_{1–387} prey vectors were described previously (37, 41, 42). Quantitative assays for β -galactosidase activity were performed as described (43), except that yeast cells were grown in minimal medium supplemented with 2% galactose, 1% raffinose, and lacking uracil, histidine, and tryptophan. To test their effect on protein-protein interactions, PP1 analogs or an equal volume of DMSO as control, were added to the liquid growth media at the indicated concentrations. Expression in yeast of LexA fusion proteins was determined by Western blot analysis using LexA antibodies (Upstate) and chemiluminescent visualization, as previously described (35).

Agrobacterium-mediated Transient Expression—Expression cassettes containing Pto forms driven by the CaMV 35S promoter and followed by the Nos terminator were excised by the EcoRI restriction enzyme from the pTEX shuttle vector and inserted at the corresponding site of the binary vector pBTEX (35). pBTEX plasmids were introduced into *Agrobacterium* GV2260 by electroporation. Before inoculation, *Agrobacterium* strains were grown overnight at 28 °C in Luria broth medium with the appropriate antibiotics and washed in infiltration medium (10 mM morpholinoethanesulfonic acid, 10 mM MgCl₂ and 0.2 mM acetosyringone). For co-expression of Pto forms with AvrPto or AvrPtoB, *Agrobacterium* strains expressing Pto forms were mixed with strains carrying the plasmids pBTEX:avrPto (35) or pBTEX:avrPtoB (24) at a final A₆₀₀ = 0.06 each, and infiltrated into leaves of 6–8-week-old *N. benthamiana* or tomato (*S. lycopersicum*) plants. The tomato cultivars used in this study are Rio Grande PtoS (RG-PtoS; *pto/pto*; *Prf/Prf*) lacking a functional *Pto* gene (11), and Rio Grande *prf3* (RG-*prf3*; *Pto/Pto*; *prf/prf*) carrying a mutation in the *Prf* gene (45).

To test the effect of the PP1 analogs *in planta*, *N. benthamiana* leaves were infiltrated with *Agrobacterium* carrying the indicated gene constructs in the pBTEX binary vector and were treated 20 h after infiltration with a solution containing 100 μM 3MB-PP1, or DMSO as control, and 0.025% Silwet-77. The solution was applied using a fine brush. The HR developed within 48–72 h after infiltration, and the severity of the symptoms was recorded with scores from 1 to 5 (where 1 = no symptoms and 5 = cell death in the whole infiltrated area). An HR index reflecting the degree of symptoms developed by each Pto mutant in the different treatments, as compared with wild-type Pto, was calculated according to Equation 1 (23).

$$\left[\frac{\sum(\text{PtoM} + \text{AvrPto}) - \sum(\text{AvrPto})}{\sum(\text{Pto} + \text{AvrPto}) - \sum(\text{AvrPto})} \right] \quad (\text{Eq. 1})$$

In this formula, the entries within the brackets are the scores for symptoms caused by the expression of the indicated proteins, and PtoM represents the specific mutant tested.

RESULTS

Pto^{as} Kinase Activity Is Specifically and Potently Inhibited by ATP-competitive Small Molecules—We have previously reported the engineering of an analog-sensitive form of Pto (Pto^{as}), which retains catalytic activity despite containing an enlarged ATP-binding site (34). Because of the enlarged ATP-binding pocket, Pto^{as} is predicted to be sensitized to specific inhibition by ATP-competitive small molecules, such as analogs of the kinase inhibitor PP1 (46). In this study, we set out to use a combination of Pto^{as} and specific small molecule inhibitors to assess the role of Pto kinase activity in recognition of *Pst* effectors and signal transduction.

To identify specific and potent small molecule inhibitors of Pto^{as} kinase activity, we screened a panel of various PP1 analogs (supplemental Fig. S1) for their ability to inhibit *in vitro* Pto^{as} and wild-type Pto autophosphorylation and phosphorylation of the substrate Pti1(K96N). As shown in Fig. 1A, all the inhibitors examined, with the exception of 2NM-PP1 and aMB-PP1, specifically inhibited the ability of Pto^{as} to phosphorylate Pti1(K96N), while having negligible effect on wild-type Pto. Similar results were obtained for Pto^{as} and wild-type Pto autophosphorylation (data not shown). IC₅₀ values were then determined for analogs that at a concentration of 1 μM showed over 75% inhibition of Pto^{as} kinase activity (3MB-PP1, 1NM-PP1, 2Na-PP1, and Ac-PP1). 1NM-PP1 and 3MB-PP1 were the most potent inhibitors with IC₅₀ values of 120 and 160 nM, respectively (Table 1).

To further confirm the specificity of 1NM-PP1 and 3MB-PP1 to Pto^{as}, we tested *in vitro* the ability of these PP1 analogs to inhibit activity of the tomato serine/threonine kinase Pti1 and of the MAP kinase MPK2, which were previously proposed to participate in signaling pathways downstream of Pto (20, 37). The effect of the two inhibitors was tested at concentrations corresponding to their IC₅₀ values for Pto^{as} and at 1 μM . As shown in Fig. 1B, 1NM-PP1 and 3MB-PP1 were not able to significantly inhibit Pti1 and MPK2. Because of their potency and specificity to Pto^{as}, these ATP-competitive small molecule inhibitors were selected for subsequent functional analysis of Pto.

Pto^{as} Displays Functional Properties of Wild-type Pto—Functional properties of Pto include its ability to elicit the HR when co-expressed with the *Pst* effectors AvrPto or AvrPtoB in *N. benthamiana* and tomato plants, respectively (24, 41). In addition, Pto physically interacts with AvrPto and AvrPtoB in a yeast two-hybrid system (24, 41). To validate the use of Pto^{as} in the present study, we tested whether this Pto variant displays functional properties similar to wild-type Pto. The competence of Pto^{as} to elicit the HR was assessed using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* plants and in the tomato line RG-PtoS that do not contain a recognition determinant for AvrPto and AvrPtoB, respectively (24, 47). Co-expression of Pto^{as} and AvrPto in *N. benthamiana*, or co-expression of Pto^{as} and AvrPtoB in RG-PtoS caused the devel-

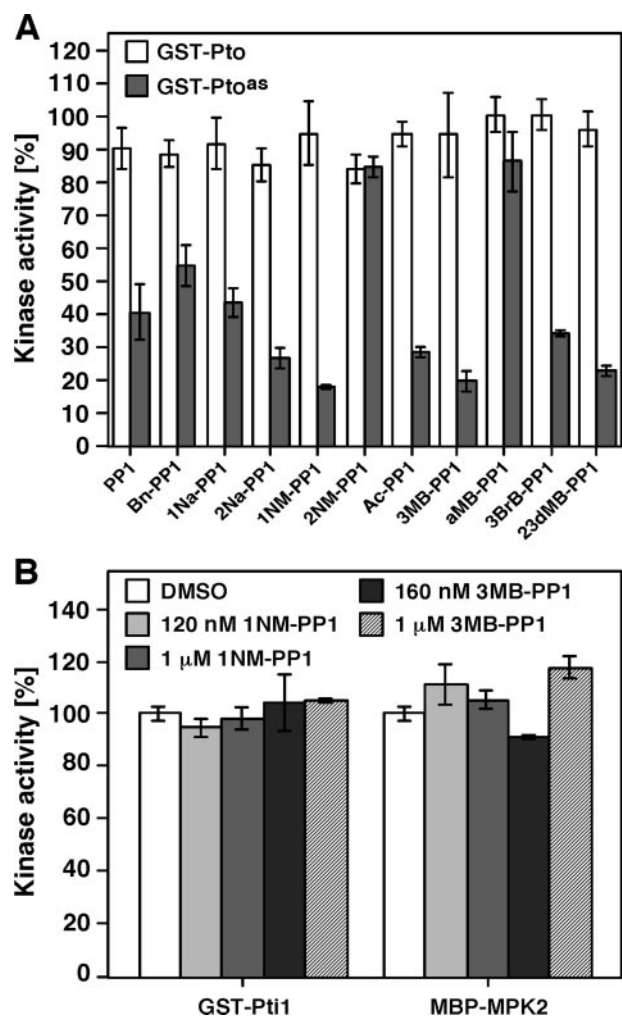


FIGURE 1. PP1 analogs specifically inhibit Pto^{as} kinase activity. *A*, *in vitro* phosphorylation of the kinase-deficient mutant GST-Pti1(K96N) by GST-Pto (white bars) and GST-Pto^{as} (gray bars) in the presence of [γ -³²P]ATP and the indicated PP1 analogs (1 μ M). Kinase activity in the presence of inhibitors is measured as percentage of that observed in the absence of inhibitors. Data are the means of three technical repeats \pm S.E. The assay was repeated at least two times for each inhibitor with similar results. *B*, *in vitro* autophosphorylation of GST-Pti1 and MBP-MPK2 in the presence of [γ -³²P]ATP and the indicated PP1 analogs at 1 μ M or at IC₅₀ values determined for GST-Pto^{as} activity, as indicated in Table 1. Kinase activity in the presence of inhibitors is measured as percentage of that observed in the absence of inhibitors (DMSO). Data are the means of three technical repeats \pm S.E.

TABLE 1
50% inhibitory concentration (IC₅₀) of selected PP1 analogs for Pto^{as} kinase activity

Inhibition assays were carried out by testing GST-Pti1(K96N) phosphorylation by GST-Pto^{as} in the presence of various inhibitor concentrations ranging from 1 nM to 1 μ M.

Inhibitor	IC ₅₀ ^a
	nM
1NM-PP1	120
3MB-PP1	160
2Na-PP1	250
Ac-PP1	350

^a IC₅₀ is defined as the concentration of the inhibitor at which Pto^{as} kinase activity was 50% of the control (no inhibitor).

opment of a typical HR in the inoculated areas within 48 h (Fig. 2, *A* and *B*). The appearance of the HR elicited by co-expression of Pto^{as} with AvrPto or AvrPtoB was similar in timing and intensity to that caused by expression of wild-type Pto in com-

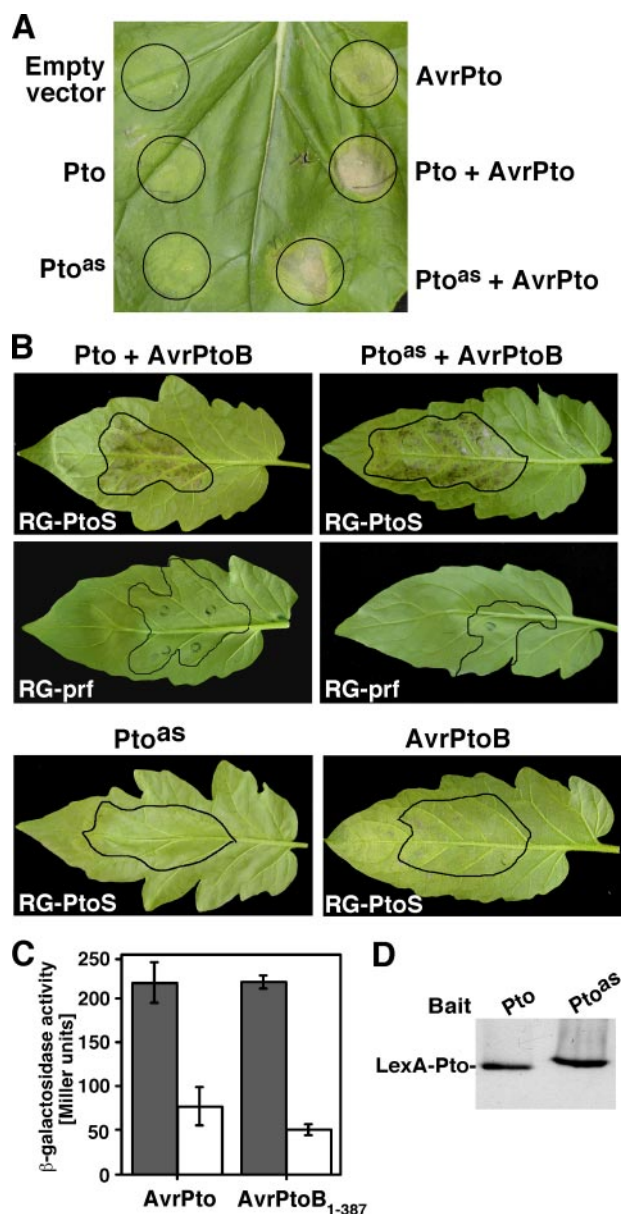


FIGURE 2. Pto^{as} is functional in the elicitation of the HR and interacts with AvrPto and AvrPtoB₁₋₃₈₇. *A* and *B*, elicitation of the HR by Pto^{as}. *N. benthamiana* (*A*) or RG-PtoS and RG-prf3 tomato leaves (*B*) were infiltrated in the encircled areas with *Agrobacterium* strains for the expression of the indicated proteins. Leaves were photographed 72 h after inoculation. Tissue collapse in the infiltrated areas reflects elicitation of the HR. *C*, physical interactions of Pto (gray bars) and Pto^{as} (white bars) with AvrPto and AvrPtoB₁₋₃₈₇ measured in a yeast two-hybrid system. Data are the means of three independent yeast transformants \pm S.E. The assay was repeated three times with similar results. *D*, expression of bait proteins in yeast. Pto and Pto^{as} were expressed in yeast as LexA fusions and visualized in immunoblots with anti-LexA antibodies.

ination with the same effectors. Leaf areas expressing either Pto, Pto^{as}, AvrPto, or AvrPtoB alone exhibited no response. In addition, the HR caused by co-expression of Pto and AvrPtoB in tomato was dependent on a functional Pto pathway because it was not induced in RG-prf3 plants carrying a mutation in the *Prf* gene, which is required for Pto signaling (45) (Fig. 2*B*).

Next, a yeast two-hybrid system was used to test the ability of Pto^{as} to physically interact with AvrPto or with the N-terminal domain of AvrPtoB (AvrPtoB₁₋₃₈₇), which is sufficient to interact with Pto and to elicit Pto-dependent immunity (42). The

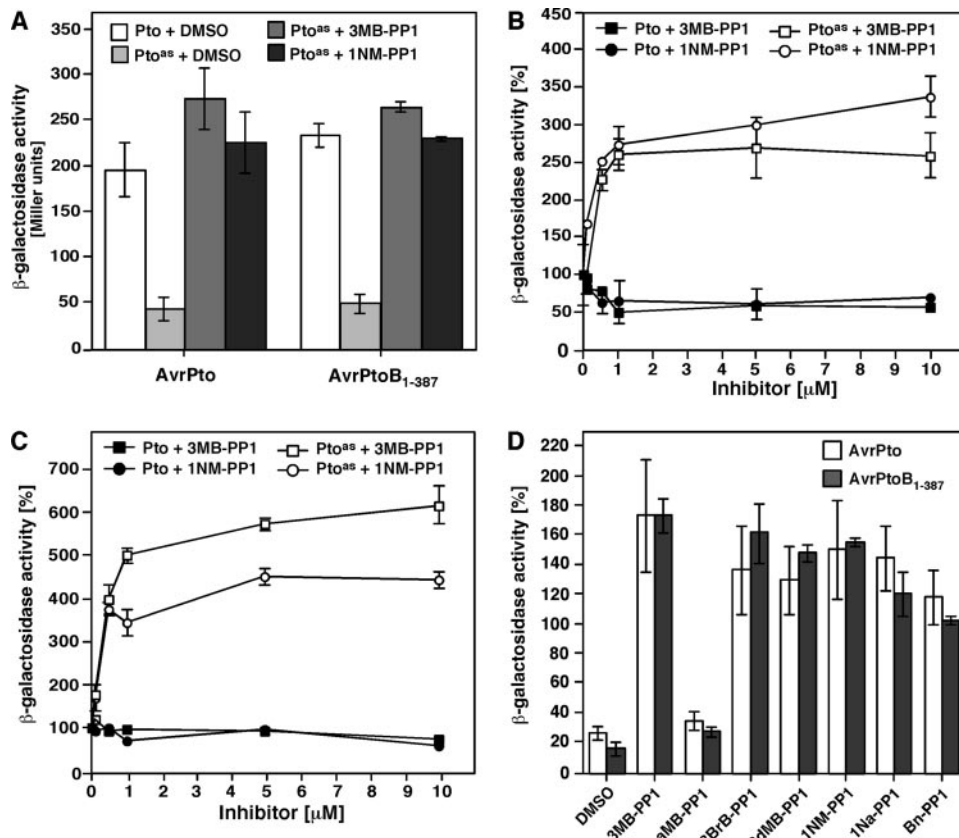


FIGURE 3. PP1 analogs enhance interactions of Pto^{as} with AvrPto and AvrPtoB₁₋₃₈₇. A, interactions of Pto and Pto^{as} with AvrPto or AvrPtoB₁₋₃₈₇ measured in a yeast two-hybrid system in the presence of 3MB-PP1 (10 μ M), 1NM-PP1 (10 μ M) or DMSO using the β -galactosidase assay. B and C, dose-response effect of 3MB-PP1 and 1NM-PP1 on the interaction of Pto and Pto^{as} with AvrPto (B) or AvrPtoB₁₋₃₈₇ (C). β -Galactosidase activity is reported as percentage of the activity observed for each bait-prey combination in the presence of DMSO. D, interactions of Pto^{as} with AvrPto (white bars) or AvrPtoB₁₋₃₈₇ (gray bars) in the presence of the indicated PP1 analogs (10 μ M). Activity is reported as percentage of that observed for the interaction of Pto with AvrPto or AvrPtoB₁₋₃₈₇ in the presence of the same analog. In each panel, data are the means of three independent yeast transformants \pm S.E. Assays were repeated at least three times with similar results.

variable expression of full-length AvrPtoB in yeast hindered its use in these experiments (data not shown and Ref. 12). As shown in Fig. 2C, Pto^{as} interacted with AvrPto and AvrPtoB₁₋₃₈₇, albeit at lower levels than wild-type Pto. Pto and Pto^{as} bait fusion proteins accumulated in yeast at similar levels excluding the possibility that the weaker interactions of Pto^{as} with effectors was a result of reduced Pto^{as} expression (Fig. 2D). Together, these results indicate that Pto^{as} retains functional properties of wild-type Pto, although it displays weaker physical interactions with *Pst* effectors.

PP1 Analogs Enhance Physical Interactions of Pto^{as} with AvrPto and AvrPtoB₁₋₃₈₇—The reduced interactions of Pto^{as} with AvrPto and AvrPtoB₁₋₃₈₇ correlated well with a 70% decrease in catalytic activity previously observed for Pto^{as} relative to wild-type Pto (34). In an attempt to assess whether kinase activity is required for the interaction of Pto with *Pst* effectors, we monitored the physical interaction of Pto^{as} with AvrPto or AvrPtoB₁₋₃₈₇ in a yeast two-hybrid system in the presence of the two potent and specific inhibitors of Pto^{as} kinase activity identified *in vitro*, 3MB-PP1 and 1NM-PP1. Unexpectedly, the addition of either 3MB-PP1 or 1NM-PP1 did not inhibit the interactions of Pto^{as} with the *Pst* effectors (Fig. 3A), even at a concentration of 10 μ M that completely inhibited

kinase activity of Pto^{as} *in vitro* (Fig. 1 and data not shown), and blocked the activity of typical analog-sensitized kinases in yeast (29, 48). Instead, 3MB-PP1 and 1NM-PP1 significantly potentiated the interactions of Pto^{as} with AvrPto and AvrPtoB₁₋₃₈₇ (Fig. 3A). Dose-response curves revealed that the increase in interactions reaches saturation at a drug concentration of \sim 1 μ M (Fig. 3, B and C). Interestingly, at saturation levels the strength of Pto^{as} interactions with both effectors approached that of wild-type Pto (Fig. 3, A–C). The ATP-competitive small molecules did not increase the interactions of wild-type Pto with the *Pst* effectors and the binding potentiation effect was thus specific for the drug-sensitized mutant (Fig. 3, A–C).

To test whether additional PP1 analogs have a similar effect to 3MB-PP1 and 1NM-PP1 on Pto^{as}, we screened structurally similar compounds (supplemental Fig. S1) for their ability to affect the interactions of Pto^{as} with AvrPto and AvrPtoB₁₋₃₈₇. As shown in Fig. 3D, all the PP1 analogs tested, with the exception of aMB-PP1, enhanced the interactions of Pto^{as} with both *Pst* effectors to levels comparable with those of wild-type Pto. The

ability of these PP1 analogs to affect Pto^{as}-effector interactions correlated well with the potency of their inhibition against Pto^{as} *in vitro* kinase activity (Fig. 1). This strongly supports the notion that the binding of PP1 analogs to the active site of Pto^{as} underlies their potentiating effects on Pto^{as}-effector interactions.

Kinase Activity Is Dispensable for Pto^{as} Interactions with AvrPto and AvrPtoB₁₋₃₈₇ in the Presence of PP1 Analogs—A positive rather than negative effect of the PP1 analogs on Pto^{as} protein-protein interactions suggested that in their presence Pto^{as} does not require kinase activity to interact with AvrPto or AvrPtoB₁₋₃₈₇. However, it remains possible that the residual level of Pto^{as} kinase activity in the presence of the inhibitors is sufficient to carry out the phosphorylation required for Pto-effector interactions. To directly test this possibility, we generated kinase-deficient forms of Pto^{as} by further mutating catalytically essential residues in the kinase. To this end, mutations were introduced in Pto^{as} at the invariant residue Lys-69 of the kinase subdomain II, which is involved in ATP binding, or at the conserved Asp-182 of the DFG motif in the kinase subdomain IV, which is required for binding of divalent cations (49). As expected, Pto^{as}(K69R) and Pto^{as}(D182E) did not autophosphorylate or phosphorylate the Pto substrate Pt1(K96N) in a kinase assay *in vitro* (Fig. 4A) and lost their ability to interact with

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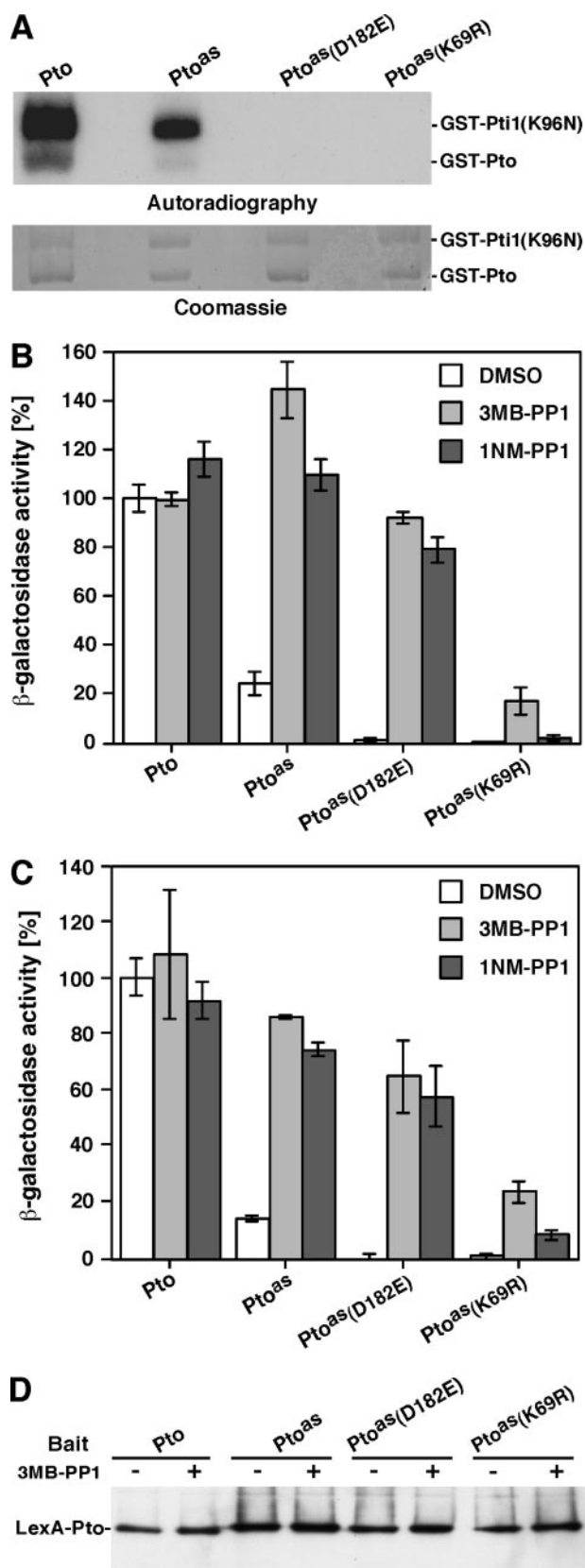


FIGURE 4. 3MB-PP1 and 1NM-PP1 drugs rescue the interactions of Pto^{as} kinase-deficient forms with AvrPto and AvrPtoB₁₋₃₈₇. *A*, kinase activity assay of Pto variants. Autophosphorylation and phosphorylation of GST-Pti1(K96N) by the indicated GST-Pto forms was tested *in vitro*, and proteins were fractionated by 10% SDS-PAGE. The gel was stained by Coomassie Blue (bottom panel) and exposed to autoradiography (top panel). *B* and *C*, interac-

AvrPto and AvrPtoB₁₋₃₈₇ in a yeast two-hybrid system (Fig. 4, *B* and *C*). However, the addition of 3MB-PP1 or 1NM-PP1 to the yeast growth medium rescued the interactions of the Pto^{as} kinase-deficient variants with both *Pst* effectors (Fig. 4, *B* and *C*). In the presence of 3MB-PP1 and 1NM-PP1, the interaction of Pto^{as}(D182E) with either AvrPto or AvrPtoB₁₋₃₈₇ was ~80 and 60% of wild-type Pto, respectively. Interactions of Pto^{as}(K69R) with the two *Pst* effectors were only partially restored by the PP1 analogs to 10–20% of the wild-type level (Fig. 4, *B* and *C*). These results suggest that the binding of ATP-competitive small molecules to Pto^{as} renders the kinase activity dispensable for its interactions with AvrPto or AvrPtoB₁₋₃₈₇.

To exclude the possibility of an indirect effect of the PP1 analogs on the expression and stability of Pto variants in yeast, protein levels of the various Pto bait forms were determined by immunoblot analysis using antibodies raised against the LexA fusion. As shown in Fig. 4*D*, steady-state levels of all Pto forms were not affected by the presence of 3MB-PP1 at a concentration of 10 μM, which was the highest concentration used in the yeast two-hybrid experiments.

*PP1 Analogs Bypass Pto^{as} Autophosphorylation at Thr-199 and Their Effect Is Dependent on a Pto-AvrPto Natural Interface—*Autophosphorylation at Pto Thr-199 was previously shown to be important for Pto recognition of AvrPto and was proposed to be necessary for stabilization of the Pto P+1 loop (23, 27). To examine whether the effect of autophosphorylation at Pto Thr-199 is also bypassed by PP1 analogs, we generated the mutant Pto^{as}(T199A) and tested its interactions with AvrPto and AvrPtoB₁₋₃₈₇. Pto^{as}(T199A) was severely impaired in its ability to bind AvrPto and AvrPtoB₁₋₃₈₇ (Fig. 5, *A* and *B*), while still retained a similar kinase activity *in vitro* as Pto^{as} and Pto(T199A) (Fig. 5*C*). However, the addition of either 3MB-PP1 or 1NM-PP1 to the yeast growth medium completely rescued the ability of Pto^{as}(T199A) to interact with AvrPto and AvrPtoB₁₋₃₈₇, whereas it did not affect the interactions of Pto(T199A) with the *Pst* effectors (Fig. 5, *A* and *B*). Immunoblots performed using anti-LexA antibody confirmed that steady-state levels of Pto mutants in yeast were not affected by the presence of 10 μM 3MB-PP1 (Fig. 5*D*). These results suggest that the effect of autophosphorylation at Thr-199 on Pto-effector interactions can be bypassed by binding of PP1 analogs to the enlarged Pto^{as} ATP-binding site.

The recent crystal structure determination of the Pto-AvrPto complex revealed that a main interface that mediates the interaction of the two proteins is between the P+1 loop of Pto and the GINP motif of AvrPto (27). In addition, introduction of mutations in the GINP motif of AvrPto hampers the interaction of the mutant effector with Pto in a yeast two-hybrid system (50). To verify that the ability of Pto^{as} to bind AvrPto in the

tions of Pto forms with AvrPto (*B*) or AvrPtoB₁₋₃₈₇ (*C*) measured in a yeast two-hybrid system in the presence of 3MB-PP1 (10 μM), 1NM-PP1 (10 μM), or DMSO using the β-galactosidase assay. β-Galactosidase activity is reported as percentage of that observed for the interaction of Pto with AvrPto or AvrPtoB₁₋₃₈₇ in the presence of DMSO. Data are the means of three independent yeast transformants ± S.E. The assay was repeated three times with similar results. *D*, expression of bait proteins in yeast in the presence or absence of 10 μM 3MB-PP1. Wild-type and mutant forms of Pto were expressed in yeast as LexA fusions and visualized in immunoblots with anti-LexA antibodies.

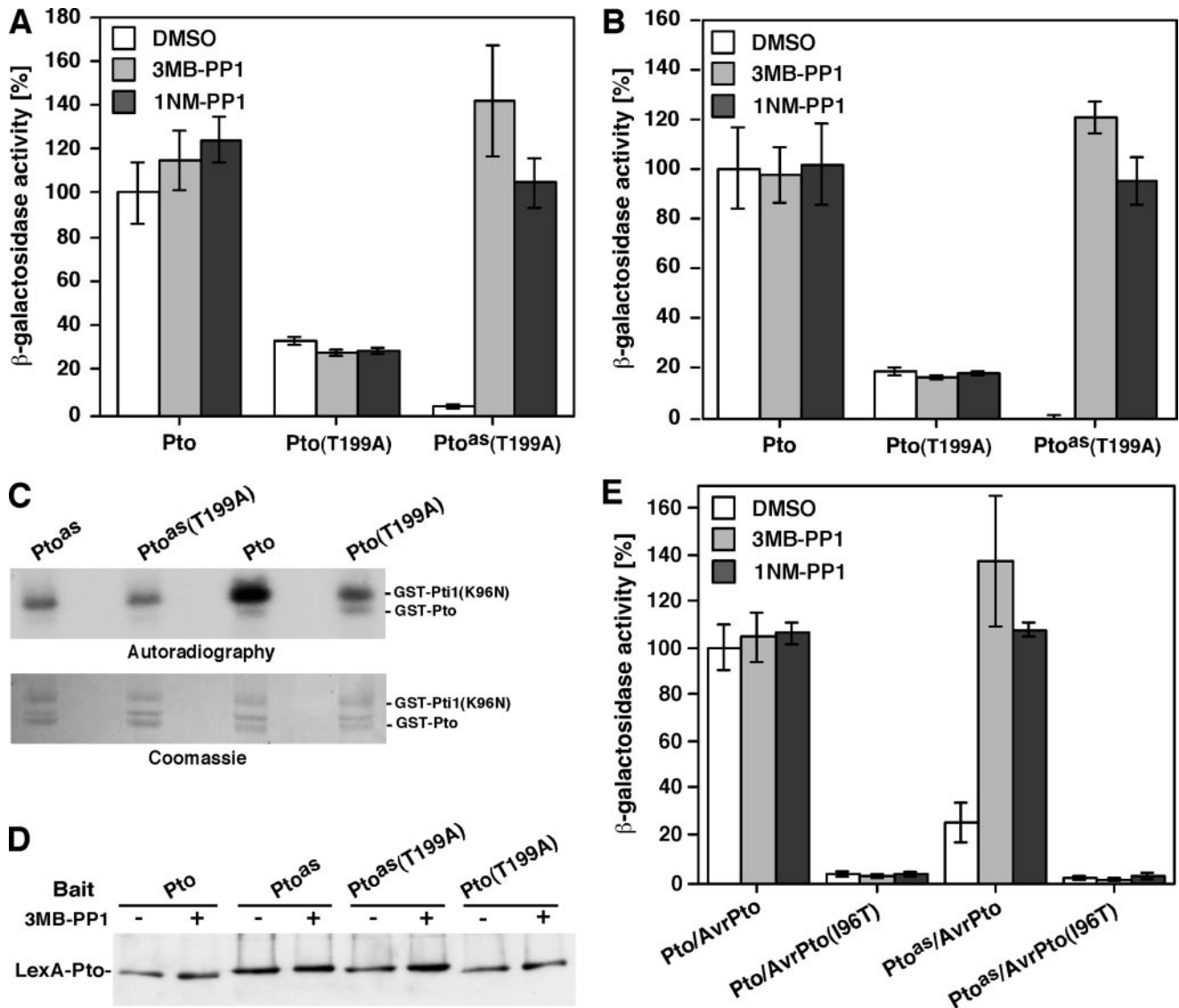


FIGURE 5. 3MB-PP1 and 1NM-PP1 rescue the interactions of Pto^{as}(T199A) with bacterial effectors, but not the interaction of Pto^{as} with AvrPto(I96T). A and B, interactions of Pto forms with AvrPto (A) or AvrPtoB₁₋₃₈₇ (B) measured in a yeast two-hybrid system in the presence of 3MB-PP1 (10 μ M), 1NM-PP1 (10 μ M), or DMSO using the β -galactosidase assay. β -Galactosidase activity is reported as percentage of that observed for the interaction of Pto with AvrPto or AvrPtoB₁₋₃₈₇ in the presence of DMSO. Data are the means of three independent yeast transformants \pm S.E. The assay was repeated three times with similar results. C, kinase activity assay of Pto forms. Autophosphorylation and phosphorylation of GST-Pti1(K96N) by the indicated GST-Pto forms was tested *in vitro*, and proteins were fractionated by 10% SDS-PAGE. The gel was stained by Coomassie Blue (bottom panel) and exposed to autoradiography (top panel). D, expression of bait proteins in yeast in the presence or absence of 10 μ M 3MB-PP1. Wild-type and mutant forms of Pto were expressed in yeast as LexA fusions and visualized in immunoblots using anti-LexA antibodies. E, interactions of Pto and Pto^{as} with AvrPto or AvrPto(I96T) in the presence of 3MB-PP1 (10 μ M), 1NM-PP1 (10 μ M), or DMSO. Data are the means of three independent yeast transformants \pm S.E. The assay was repeated three times with similar results.

presence of ATP-competitive drugs is mediated by this natural interface, we tested the interaction of Pto^{as} with the GINP motif mutant AvrPto(I96T) in the yeast two-hybrid system. As expected, both wild-type Pto and Pto^{as} were unable to interact with AvrPto(I96T) (Fig. 5E). Addition of 3MB-PP1 or 1NM-PP1 to the yeast growth medium did not rescue these interactions. These results indicate that rescue of the Pto^{as}-AvrPto interaction by PP1 analogs is dependent on and occurs through a natural interaction interface between Pto and AvrPto.

Kinase Activity Is Dispensable for Pto Signaling in Planta—We next investigated the requirement of Pto kinase activity for effector-dependent signaling *in planta* by exploiting the ability

of PP1 analogs to bypass the requirement of kinase activity for the interactions of Pto with bacterial effectors. Specifically, we tested whether expression of AvrPto and kinase-deficient forms of Pto^{as} is sufficient for elicitation of the HR in the presence of PP1 analogs. To this aim, Pto, Pto^{as}, and its kinase-deficient variants Pto^{as}(D182E) and Pto^{as}(K69R) were each co-expressed with AvrPto in *N. benthamiana* leaves using an *Agrobacterium*-mediated transient expression assay. Twenty hours after infiltration of *Agrobacterium* suspensions, leaves were treated with either 100 μ M of 3MB-PP1 or DMSO as control, and monitored for the appearance of tissue collapse and cell death that is characteristic of the HR. Finally, to estimate the extent of cell death, an HR index was calculated for each

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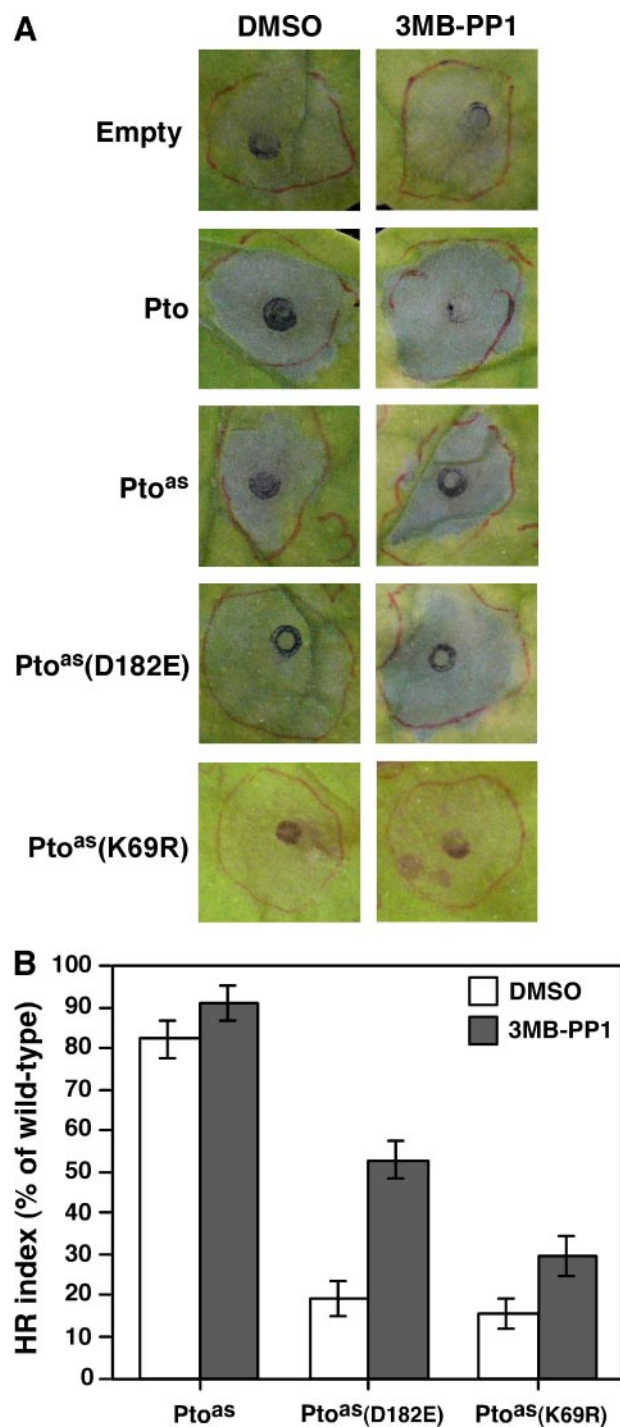


FIGURE 6. Elicitation of the HR by expression of Pto^{as} mutants and AvrPto in *N. benthamiana* leaves in the presence of 3MB-PP1. *A*, *N. benthamiana* leaves were infiltrated with *Agrobacterium* strains for the expression of either AvrPto and the indicated Pto form or AvrPto alone (*empty*). Infiltrated areas were treated 20 h after infiltration with 3MB-PP1 or DMSO as control. The HR was visualized as localized tissue collapse typically within 2–3 days. *B*, HR index reflecting the efficiency of the indicated Pto^{as} form and treatment (3MB-PP1 or DMSO) in the elicitation of the HR. The HR index was calculated as described under “Experimental Procedures” and included at least 51 independent observations for each mutant. Data are the means \pm S.E.

Pto^{as} variant and treatment (DMSO or 3MB-PP1) and normalized to that of wild-type Pto.

As expected, in the presence of DMSO or 3MB-PP1, expression of AvrPto and Pto elicited a typical HR (Fig. 6*A*). Similarly,

Pto^{as} was functional in elicitation of the HR, although its HR index was 20 and 10% lower than that of wild-type Pto in the presence of DMSO and 3MB-PP1, respectively (Fig. 6, *A* and *B*). In contrast, the kinase-deficient variants Pto^{as}(D182E) and Pto^{as}(K69R) were severely impaired in induction of the HR in the presence of DMSO (Fig. 6, *A* and *B*). Remarkably, addition of 3MB-PP1 significantly recovered the HR-inducing ability of Pto^{as}(D182E) to \sim 50% of wild-type Pto, while it caused a milder increase in the HR index of Pto^{as}(K69R) to \sim 30% (Fig. 6, *A* and *B*). The quantitatively different effect of 3MB-PP1 on the HR-inducing activity of the two kinase-deficient Pto^{as} variants well correlated with the differential ability of the drug to restore their physical interactions with AvrPto and AvrPtoB_{1–387} (Fig. 4, *B* and *C*). It should be noted that the rescue effect of 3MB-PP1 on Pto^{as}(D182E) and Pto^{as}(K69R) was absolutely dependent on the expression of AvrPto (supplemental Fig. S2), thus excluding the possibility that this drug renders the Pto^{as} kinase-deficient variants constitutively active and independent of AvrPto activation. Together, these results suggest that following the physical interaction between Pto and AvrPto, Pto kinase activity is dispensable for Pto signaling *in planta*.

DISCUSSION

We used a chemical-genetic strategy to develop small molecule inhibitors that specifically target the kinase activity of the tomato R protein Pto. These highly selective inhibitors allowed us to shed new light on the controversial role of Pto kinase activity in recognition of *Pst* effectors and signal transduction. The results of this study, interpreted in combination with structural data (27), suggest a novel form of protein kinase regulation: kinase activity is required to stabilize Pto in the proper conformation for interacting with the AvrPto and AvrPtoB bacterial effectors, but is dispensable for Pto-mediated signal transduction.

Small molecule inhibitors are widely used for the functional study of animal protein kinases, but very few pharmacological tools are available for plant protein kinases. The challenge of inhibitor specificity is more pronounced in the plant kinases since there are >1,000 kinases in plant genomes compared with roughly half the number in human and mouse genomes (1, 2, 51). To develop the first specific inhibitor of Pto, we utilized a chemical-genetic approach to inhibitor development that has been widely and successfully applied to examine cellular functions of several kinases from different organisms and revealed kinase-dependent and -independent functions of these proteins (29, 31–33, 52). An important advantage of this approach is that Pto^{as} retains molecular properties qualitatively similar to those of wild-type Pto. At the same time, Pto^{as} kinase activity can be rapidly and reversibly inactivated by cell-permeable small molecule inhibitors. In contrast, other approaches, which were previously used for addressing the role of kinase activity for Pto function, involved mutants that are irreversibly impaired in one or more molecular properties, thus complicating interpretation of the results. For example, Pto mutants in kinase catalytic residues are kinase-deficient, most of them do not interact with AvrPto and are not functional in the elicitation of the HR (25, 41, 47). Similarly, constitutively active Pto

forms that activate an effector-independent HR do not interact with effectors and are impaired in kinase activity (25, 26).

Potent and specific inhibitors of Pto^{as} selected from a panel of PP1 analogs allowed us to examine the role of kinase activity in Pto recognition of the AvrPto and AvrPtoB effectors. Previous reports based on biochemical and structural data suggested that phosphorylation at Thr-199 in the P+1 loop of the Pto activation domain plays an important role in the recognition of bacterial effectors (23, 27). Phosphorylation at Thr-199 was proposed to stabilize Pto conformation and to represent a prerequisite for its interaction with AvrPto (27). Here we show that PP1 analogs, while efficiently inhibiting the kinase activity of the protein *in vitro*, potentiated the interaction of Pto^{as} with the bacterial effectors in a dose-dependent fashion up to wild-type Pto levels. Moreover, the inhibitors rescued the interactions of the kinase-deficient Pto^{as}(D182E) and of the P+1 loop mutant Pto^{as}(T199A) with AvrPto and AvrPtoB. However, their effect was less prominent on the interactions between the kinase-deficient Pto^{as}(K69R) and the effectors. We can speculate that this is the result of a lower affinity of the PP1 analogs to the mutant kinase due to the central position of Lys-69 in the ATP binding site as opposed to Asp-182, which is located on its outskirts (27). Thus, the ability of the small molecule ligands to bypass Pto kinase activity and activation-domain phosphorylation further supports the notion that an active conformation, rather than a docking phosphorylated residue(s), is critical for the interactions of Pto with effectors.

The molecular mechanism that allows PP1 analogs to potentiate the interaction of Pto^{as} with bacterial effectors remains to be determined. We first hypothesized that PP1 analogs could affect Pto^{as} stability or create a novel unnatural interface between Pto^{as} and effectors. However, these possibilities are unlikely because 3MB-PP1 did not affect Pto^{as} steady-state levels in yeast and the effect of PP1 analogs on the Pto^{as}-AvrPto interaction was dependent on a natural interface. An alternative scenario is that PP1 analogs induce in Pto^{as} a conformational change that leads to stabilization of the activation loop mimicking the effect of phosphorylation at Thr-199 in wild-type Pto. This hypothesis is supported by the evidence that PP1 analogs bypass the need of phosphorylation at Thr-199 for a proper Pto-AvrPto interaction. Finally, the possibility that PP1 analogs stimulate interaction with effectors by mimicking the ATP-bound form of the kinase should also be considered, although this is less likely based on the evidence that Pto^{as}(T199A) displays a very weak interaction with effectors while still retaining kinase activity, which implies ATP binding.

Bypassing the need of kinase activity with ATP-competitive small molecules is not unprecedented. In a study aimed at the dissection of its kinase function, the yeast ER stress sensor Ire1 was sensitized to inhibition of PP1 derivatives (33). Interestingly, in the presence of its kinase domain ligand 1NM-PP1, the sensitized Ire1 bypassed mutations that inactivate its kinase activity and was fully functional in the activation of unfolded protein response (33). The effect of 1NM-PP1 on the sensitized Ire1 was attributed to a conformational change and/or to a change in the oligomeric state of the enzyme similar to that caused by binding of ADP to the wild-type kinase (33). In the case of Ire1, it contains an endonuclease domain in addition to the kinase domain. The endonuclease activ-

ity mediates the majority of the signaling output from Ire1 while the kinase activity primarily modulates the endonuclease activity. In contrast, Pto contains only the catalytic activity of a protein kinase and is a monoenzyme. Our results that binding of a small-molecule ligand could bypass catalytic activity of a monoenzyme kinase argue against the stereotypic view of protein kinases relying on their enzymatic activity for signaling output. The effect of PP1 analogs on Pto^{as} and Ire1^{as} might be part of a wider phenomenon that could apply to other protein kinases. Understanding the structural basis of this phenomenon may lead to the identification of additional kinases that can be switched to an active conformation by small molecule ligands, bypassing the need of their kinase activity. This may allow the elaboration of strategies for the chemical regulation of certain protein kinase functions by small molecules that selectively bind to their kinase ATP-binding site.

The effect of PP1 analogs on Pto-effector interactions was specific for Pto^{as} and independent on the identity of effector protein. This confirms that the conformational requirements of Pto for the recognition of AvrPto and AvrPtoB are similar, in agreement with previous reports showing that different Pto variants, including chimeras of Pto with the related kinase Fen, mutants in autophosphorylation sites and surface-exposed residues, interacted with AvrPto and AvrPtoB with similar specificity (24, 26).

By a combination of small molecule ligands directed at the kinase active site and a kinase-deficient form of Pto^{as} we provide evidence supporting the notion that kinase activity is dispensable for signaling by Pto *in planta*. In fact, bypassing the requirement of Pto kinase activity for the interaction with AvrPto was sufficient for a Pto^{as} kinase-deficient form to trigger an AvrPto-dependent HR in *N. benthamiana* plants. This is the first time that a kinase-deficient variant of Pto is shown to activate an effector-dependent HR and demonstrates that PP1 analogs can be used *in planta* for the pharmacological regulation of Pto functions. Our data are consistent with a conformational change induced in Pto by AvrPto, switching it from an inactive to an active state. In addition, they confirm that the inhibitory effect on Pto kinase activity that was recently shown for AvrPto *in vitro* (27) does not play a role in Pto activation.

The molecular mechanisms used by Pto to relay the signal conveyed by effector recognition are still unknown. Early studies proposed that Pto initiates signaling pathways by phosphorylation of downstream proteins (37, 44, 53). Although *in vitro* substrates of Pto phosphorylation have been identified, there is no direct evidence *in vivo* that corroborates this hypothesis. More recently, Pto has been shown to interact *in vivo* with the NBS-LRR protein Prf in a high molecular weight complex (19). The Pto-Prf interaction and additional genetic and molecular data support a model in which Pto signals by regulating Prf function (19, 27). Our findings that kinase-deficient Pto^{as} activates an effector-dependent HR in the presence of PP1 analogs argues against the model of Pto signaling through phosphorylation of downstream proteins. In addition, they suggest that Pto transphosphorylation activity is not absolutely required in mechanisms of Pto signaling, including the proposed activation of Prf, but may play an auxiliary role. This is in agreement with previously published results showing that constitutively active Pto mutants that lack kinase activity *in vitro* induce an effector-independent but Prf-dependent HR *in planta* (25, 26). How-

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ever, it is still possible that phosphorylated residues may play a role in stabilizing the Pto-activated form. A possible candidate residue for such a role could be the Pto autophosphorylation site Ser-198, which was shown to be required for the elicitation of the HR, but dispensable for the interaction with AvrPto (23).

In conclusion, the emerging picture from results of the current study and previous investigation is that Pto kinase activity plays an important role through autophosphorylation in the stabilization of the Pto molecule in the proper conformation for interacting with bacterial effectors, but not in Pto-mediated signal transduction. Pto appears to rely on a switch between conformation states to convey the signal: the protein is first activated by an effector-induced conformational change, which is subsequently sensed by Prf to activate plant immune responses. Finally, the chemical-genetic strategy used here to develop small-molecule inhibitors that specifically target the kinase activity of Pto represents an invaluable means for the definition of biological functions of other plant kinases, for which available pharmacological tools are very limited.

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