

1 *Pseudomonas syringae* effector HopZ3 suppresses the bacterial AvrPto1–
2 tomato PTO immune complex via acetylation

3

4 Short title: PTO defense pathway in tomato is suppressed by acetylation

5

6 Joanna Jeleńska^{1¶}, Jiyoung Lee^{1,¶¶}, Andrew J. Manning¹, Donald J. Wolfgeher¹,
7 Youngjoo Ahn¹, George Walters-Marrah¹, Ivan E. Lopez¹, Lissette Garcia¹, Sheri A.
8 McClerklin¹, Richard W. Michelmore², Stephen J. Kron¹ and Jean T. Greenberg^{1*}

9

10 ¹Department of Molecular Genetics and Cell Biology, The University of Chicago,
11 Chicago, Illinois, USA

12 ²The Genome Center & Department of Plant Sciences, University of California, Davis,
13 California, USA

14 [#] Current address: Biological Resource Center, Korea Research Institute of Bioscience
15 and Biotechnology, Jeongeup, Jeollabuk-do, South Korea

16

17

18 * jgreenbe@uchicago.edu

19

20 ^{¶¶} These authors contributed equally to this work.

Abstract

The plant pathogen *Pseudomonas syringae* secretes multiple effectors that modulate plant defenses. Some effectors trigger defenses due to specific recognition by plant immune complexes, whereas others can suppress the resulting immune responses. The HopZ3 effector of *P. syringae* pv. *syringae* B728a (*Psy*B728a) is an acetyltransferase that modifies not only components of plant immune complexes, but also the *Psy* effectors that activate these complexes. In Arabidopsis, HopZ3 acetylates the host RPM1 complex and the *Psy* effectors AvrRpm1 and AvrB3. This study focuses on the role of HopZ3 during tomato infection. In *Psy*-resistant tomato, the main immune complex includes PRF and PTO, a RIPK-family kinase that recognizes the AvrPto effector. HopZ3 acts as a virulence factor on tomato by suppressing AvrPto1_{*Psy*}-triggered immunity. HopZ3 acetylates AvrPto1_{*Psy*} and the host proteins PTO, SIRIPK and SIRIN4s. Biochemical reconstruction and site-directed mutagenesis experiments suggest that acetylation acts in multiple ways to suppress immune signaling in tomato. First, acetylation disrupts the critical AvrPto1_{*Psy*}-PTO interaction needed to initiate the immune response. Unmodified residues at the binding interface of both proteins and at other residues needed for binding are acetylated. Second, acetylation occurs at residues important for AvrPto1_{*Psy*} function but not for binding to PTO. Finally, acetylation reduces specific phosphorylations needed for promoting the immune-inducing activity of HopZ3's targets such as AvrPto1_{*Psy*} and PTO. In some cases, acetylation competes with phosphorylation. HopZ3-mediated acetylation suppresses the kinase activity of SIRIPK and the phosphorylation of its SIRIN4 substrate previously implicated in PTO-signaling. Thus, HopZ3 disrupts the functions of multiple immune components and the effectors that trigger them, leading to

increased susceptibility to infection. Finally, mass spectrometry used to map specific acetylated residues confirmed HopZ3's unusual capacity to modify histidine in addition to serine, threonine and lysine residues.

Author Summary

By secreting virulence proteins (effectors) into their hosts, pathogenic bacteria hijack host cellular processes to promote bacterial colonization and disease development. For the plant pathogen *Pseudomonas syringae*, the coordinated action of effectors often mediates modifications of host defense proteins to inhibit their function. However, plants have evolved the ability to induce innate immunity upon recognition of effector-induced modifications of host proteins. How do pathogens circumvent the immune-inducing activity of certain effectors? They deploy more effectors to suppress these defenses. HopZ3, an acetyltransferase from *P. syringae*, is unique among plant pathogen effectors characterized so far in its ability to modify not only multiple components of the effector-triggered immune pathway, but also the triggering effector itself. Through the direct acetylation of residues involved in the interaction and activation of the bacterial effector AvrPto1_{Psy} and tomato kinase PTO, HopZ3 modifications disrupt their binding and block phosphorylations necessary for immune induction. Additionally, HopZ3 acetylates other possible components in the PTO signaling pathway, including activation sites in SIRIPK kinase, leading to suppression of its activity and reduced phosphorylation of SIRIN4s. Our study emphasizes the importance of HopZ3-dependent acetylation of immune complexes and bacterial effectors across plant species in the suppression of effector-induced immunity.

Introduction

The plant pathogen *Pseudomonas syringae* uses type III-secreted proteins to promote its growth during infection of plants. These effector proteins are injected into plant cells, where they often interfere with plant defense signaling either through binding, post-translational modifications (PTMs) and/or destabilization of host factors [1,2]. A major mechanism to suppress *P. syringae* growth is signaling mediated by plant immune receptors that monitor specific perturbations caused by effectors. A well-studied example of such a receptor is Arabidopsis RESISTANCE TO *P. SYRINGAE* MACULICOLA 1 (RPM1), a member of the NUCLEOTIDE BINDING-LEUCINE RICH REPEAT (NB-LRR) protein family. Recognition and signaling occur when RPM1 senses a specific phosphorylation (mainly p-T166) of RPM1-INTERACTING PROTEIN 4 (RIN4), an intrinsically disordered hub protein [3]. Two unrelated effectors, AvrB or AvrRpm1, from different *P. syringae* strains can strongly trigger RPM1 signaling and are thus considered avirulence factors. These effectors cause the cytoplasmic RIN4-INDUCED PROTEIN KINASE (RIPK and probably additional kinases) to phosphorylate RIN4. RIN4 is also involved in promoting defense signaling in response to conserved microbial patterns. Immune responses are induced by phosphorylations of specific RIN4 residues that are triggered by recognition of effectors or microbial patterns [3-6].

Pseudomonas syringae pv. *syringae* B728a (*Psy*B728a) is a bean pathogen that can also grow to moderate levels on Arabidopsis and tomato without causing overt disease symptoms [7,8]. In Arabidopsis, *Psy*B728a with a deletion of the type III secreted effector HopZ3 (*Psy*ΔHopZ3) causes the activation of RPM1 signaling. This occurs via two interacting effectors with homology to AvrB and AvrRpm1: AvrB3_{Psy} and

AvrRpm1_{Psy}. In the context of *Psy*ΔHopZ3 infection, both effectors are needed to activate signaling [9]. HopZ3 belongs to the YopJ acetyltransferase family that comprises several effectors from animal and plant pathogens. The acetyltransferase activity of HopZ3 is necessary for suppression of RPM1 activation in Arabidopsis and several components of the RPM1 immune-effector complex are substrates of HopZ3 [9]. HopZ3 acetylates the activation loop and active site residues of RIPK, which inhibits its ability to phosphorylate RIN4. Additionally, acetylation of RIN4 prevents its phosphorylation by RIPK. HopZ3 also acetylates residues in AvrB3 that are predicted to disrupt hydrogen bonds at the key interaction sites with RIN4. Thus, HopZ3 suppresses plant immunity through modification of both Arabidopsis and bacterial proteins that act in the same complex.

Interestingly, in a large screen for interactions between effectors and plant immune signaling proteins ([9], https://charge.ucdavis.edu/charge_db/interaction/Y2H/Y2H_interaction.php), we found that HopZ3 interacted with the resistance-inducing effector AvrPto1_{Psy} and its tomato targets, PTO-like proteins. Moreover, HopZ3 suppressed AvrPto1_{Psy}-induced cell death in *Nicotiana benthamiana* [8]. That suggested that HopZ3 may affect tomato immunity. The interaction between *Psy*B728a and tomato has not been well characterized; however, resistance to *P. syringae* pv. *tomato* has been studied in great detail. Resistant tomato lacks RPM1 but contains PSEUDOMONAS RESISTANCE AND FENTHION SENSITIVITY (PRF), an NB-LRR protein that forms complexes with the kinases PSEUDOMONAS SYRINGAE PV TOMATO RESISTANCE (PTO) and FENTHION SENSITIVITY (FEN) and recognizes effectors AvrPto and AvrPtoB from *P. syringae*

113 *pv. tomato* and other pathovars [10]. PTO, FEN and related cytoplasmic protein kinases
114 in the same family as RIPK show natural variation that affects their functional specificity
115 in promoting immunity in different tomato accessions [11]. PTO and FEN interact
116 differently with AvrPto and AvrPtoB. Both effectors can bind to PTO and elicit PRF-
117 dependent immune signaling [12-15]. In contrast, FEN can bind and be activated by
118 AvrPto if the key residue N202 (that corresponds to T204 in PTO) is substituted with
119 threonine [16]. Truncated versions of AvrPtoB (e.g., AvrPtoB₁₋₃₈₇) bind to FEN and
120 stimulate immunity; however, due to the C-terminal E3 ubiquitin ligase domain, full-
121 length AvrPtoB causes proteasome-dependent FEN degradation and does not trigger
122 FEN/PRF immunity [14]. Structure-based biochemical analysis has indicated that
123 AvrPto-PTO binding is a key step that leads to activation of PRF signaling [17]. The
124 kinase activity of PTO is important for disease resistance triggered by AvrPto [18-22].
125 PTO acts as a dimer or higher order complex together with PRF [17,22,23]. Although
126 AvrPto can inhibit PTO and other kinases [17], transphosphorylation between unbound
127 PTO molecules and those bound to AvrPto is thought to be needed for downstream
128 signaling [17,22,23].

129 Another potential player in PTO/PRF-conferred immunity is SIRIN4-1, one of
130 three RIN4-related proteins in tomato. Infection with *P. syringae pv. tomato* strain T1
131 engineered to express AvrPto causes reduction of SIRIN4 protein levels. Downregulation
132 of SIRIN4-1 using RNAi decreases the growth of strain T1 carrying AvrPto but not the
133 growth of strain T1 alone [24]. Thus, downregulation of SIRIN4-1 seems to specifically
134 enhance PTO-dependent resistance. Moreover, *N. benthamina* homologue of RIN4 was
135 found in a search for proteins proximal to AvrPto, suggesting their interaction [25].

*Psy*B728a has AvrPto and AvrPtoB homologues (AvrPto1_{*Psy*} and AvrPtoB_{*Psy*}/HopAB1, hereafter called AvrPtoB_{*Psy*}) that induce resistance in tomato. Transfer of a plasmid carrying AvrPto1_{*Psy*} to a *P. syringae* pv. *syringae* strain that lacks AvrPto and AvrPtoB (*Psy*61) confers PTO-dependent recognition, whereas plasmid-borne AvrPtoB_{*Psy*} confers some PTO-independent recognition that involves other members of PTO family [26]. AvrPto1_{*Psy*} is 88% identical at the amino acid level with AvrPto_{*Pto*} while AvrPtoB alleles share 52% identity. Both AvrPto1_{*Psy*} and AvrPtoB_{*Psy*} can interact with PTO in a yeast two-hybrid assay [26]. Consistent with these findings, PRF is a major factor that restricts the growth of *Psy*B728a on tomato [10,26].

We previously found that deletion of HopZ3 decreased the growth of *Psy* on tomato with functional PTO [7], raising the possibility that HopZ3 normally suppresses effector-triggered immunity in tomato. In this study, we investigated this hypothesis. Through genetics and biochemical reconstruction, our data point to a mechanism that involves immune suppression *via* acetylation of AvrPto1_{*Psy*}, PTO and other immunity factors.

Results

HopZ3 suppresses PTO/PRF defenses triggered by AvrPto1_{*Psy*}

*Psy*B728a has a strong epiphytic growth phase modulated by effectors [7]. *P. syringae* effectors, including AvrPto_{*Pto*}, are predominantly expressed by bacteria on a leaf surface and delivered to epidermal cells during infection, where they can induce and suppress defenses [7,27]. Deletion of HopZ3 reduced epiphytic growth of *Psy*B728a in a resistant tomato PtoR (76R), which has a functional PTO [7]. In a transient expression

assay in *N. benthamiana*, HopZ3 suppressed AvrPto1_{Psy}-induced cell death, a proxy for immune activation [7,8]. Therefore, it seemed plausible that the effect of HopZ3 on the growth of *Psy*B728a in tomato is dependent on PTO and PRF proteins needed for recognition and resistance triggered by AvrPto1_{Psy}. Bacterial growth of *Psy*B728a and *Psy*ΔHopZ3 was indistinguishable in *pto11* and *prf3* plants lacking functional PTO and PRF, respectively, indicating that the PTO/PRF pathway is needed for the effect of HopZ3 (Fig 1A). As expected, deletion of HopZ3 similarly restricted total (epiphytic + endophytic, Fig 1A and 1C) and epiphytic (Fig 1B and 1D) populations of *Psy*B728a in PtoR tomato and we tested these populations interchangeably in further experiments. The growth defect of *Psy*ΔHopZ3 was restored only when a plasmid carrying wild-type HopZ3 but not a catalytically inactive version (HopZ3_C300A) was introduced (Fig 1B). HopZ3 and HopZ3_C300A proteins in these strains are produced at the same level in *Psy*ΔHopZ3 [7]. These results suggest that enzymatically active HopZ3 suppresses PTO-mediated plant immunity in tomato.

Fig 1. HopZ3 promotes the growth of *Psy*B728a on PTO-containing tomato plants (PtoR) and suppresses defenses triggered by AvrPto1_{Psy}. Plants were spray inoculated with *Psy*B728a-derived strains at an OD₆₀₀=0.01 and total (epiphytic + endophytic) or epiphytic bacterial populations were quantified in 8 leaf discs or leaf disc washes, respectively. (A) Total bacterial populations of *Psy*B728a and *Psy*ΔHopZ3 were different in PtoR but were not statistically different in *pto11* and *prf-3* plants after 4 days (n=8, t-test *P<0.05). (B) HopZ3 (Z3), but not the catalytic mutant (Z3_C300A) complements the low growth phenotype of *Psy*ΔHopZ3 in PtoR tomato. (C-D) Deletion of AvrPto1_{Psy}

($\Delta A1$) from *Psy* Δ HopZ3 ($\Delta Z3$) restores total (C) and epiphytic (D) bacterial growth to WT (*Psy*B728a or *Psy*B728a/V) levels in PtoR tomato. (E) Deletion of *AvrPto1_{Psy}* from WT *Psy*B728a does not affect bacterial growth in PtoR tomato. (F) *AvrPto1_{Psy}* does not confer resistance in *pto11* plants, regardless of the presence of HopZ3. For (B,D,E) epiphytic bacteria were collected by leaf disc washes five (B) or four (D-E) days after inoculation. Different letters indicate significant differences in growth as assessed by ANOVA with Tukey's test ($P < 0.0002$) or Fisher's test $P < 0.0001$, $n=8$). For C and F, total bacteria were quantified 3 days after spray inoculation. Different letters indicate significant differences in growth ($n=8$, ANOVA with Tukey's test, $P < 0.05$). All experiments were repeated at least twice with similar results. Bars indicate standard errors.

A possible explanation for why PTO is needed to observe HopZ3's effect on promoting *Psy*B728a growth is that HopZ3 suppresses *AvrPto1_{Psy}* recognition. If this is true, the effect of deleting HopZ3 should be reversed when *AvrPto1_{Psy}* is also deleted. To test this hypothesis, we assessed the growth of a double mutant of *Psy*B728a that lacks both HopZ3 and *AvrPto1_{Psy}* in PtoR tomato. Both total (Fig 1C) and epiphytic (Fig 1D) populations of *Psy* Δ HopZ3 Δ *AvrPto1_{Psy}* were increased relative to *Psy* Δ HopZ3 to levels similar to WT *Psy*B728a. The effect of deleting *AvrPto1_{Psy}* was complemented when the double mutant was transformed with a plasmid carrying *AvrPto1_{Psy}* (Fig 1D). Deletion of *AvrPto1_{Psy}* in *Psy*B728a with intact HopZ3 had no effect on the growth of *Psy*B728a in PtoR tomato (Fig 1E), as previously reported [28]. *AvrPto1_{Psy}* did not confer resistance in *pto11* plants due to lack of functional PTO, regardless of the presence of HopZ3 (Fig 1F).

Altogether, our genetic analysis indicates that HopZ3 suppresses AvrPto1_{Psy}-triggered immunity during *Psy*B728a infections.

HopZ3 interacts with SIRIN4s, tomato kinases PTO, FEN, SIRIPK and effectors that target PTO

To investigate the molecular mechanisms of HopZ3 suppression of tomato immunity, we performed a screen for HopZ3 and AvrPto1_{Psy} interacting proteins using a semi-automated yeast two-hybrid analysis ([9], https://charge.ucdavis.edu/charge_db/interaction/Y2H/Y2H_interaction.php). Initial yeast experiments indicated interactions of HopZ3 with SIRIN4-1, SIRIN4-2, PTO homologous protein2 (PTH2), PTO homologous protein4 (PTH4), FEN, AvrPto1_{Psy} and AvrPtoB_{Psy}. We followed up on a subset of these proteins and also tested additional candidate proteins (S1 Fig and Table 1). Although HopZ3 and PTO did not show an interaction in the yeast two-hybrid assays ([7]; S1 Fig), they interacted in an *in vitro* pull-down assay and *in planta* bimolecular fluorescence complementation (BIFC) analysis (Table 1 and Figs 2A and S2). In addition, HopZ3 interacted with FEN, tomato RIN4 homologues (SIRIN4-1, -2 and -3), the bacterial effectors AvrPto1_{Psy} and AvrPtoB_{Psy} in *in vitro* pull-downs and *in planta* and with SIRIPK in yeast and *in planta* (Figs 2, S1 and S2 and Table 1).

Table 1. HopZ3 interacts with members of a tomato immune complex.

	Yeast two-hybrid	<i>In vitro</i> pull-down	BIFC in <i>N. benthamiana</i>

	HopZ3	AvrPto1 _{PSy}	HopZ3	AvrPto1 _{PSy}	HopZ3	AvrPto1 _{PSy}
SIPTO	-	+	+	+	+	weak
SIFEN	weak	+	+	-	weak	-
SIRIN4-1	+	weak	+	-/weak	+	+
SIRIN4-2	+	weak	+	weak	+	+
SIRIN4-3	nd	nd	+	-	+	+
SIRIPK	weak	+	nd	nd	+	+
AvrPto1 _{PSy}	+	+	+	nd	+	weak
AvrPtoB _{PSy}	+	+	+	+	+	-/weak

Interacting partners of HopZ3 and AvrPto1_{PSy} in yeast two-hybrid analysis, *in vitro* pull-down and *in planta* BIFC are shown. + indicates interaction; weak indicates weak signal; -, no interaction (no signal); nd, not determined. When interactions were tested in two directions, the stronger score is reported in the table. See also Figs 2, S1, S2 and S3 for details of interactions in different tests and additional combinations.

Fig 2. HopZ3 interacts with members of the PTO defense pathway and PTO

activating effectors. Pull-down experiments using recombinant tagged proteins were performed to assess the interaction between HopZ3 and proteins in the PTO immune pathway. GST, MBP or beads alone were used as negative controls. Proteins were detected by immunoblotting or Coomassie staining. (A) Immobilized GST-tagged FEN and PTO were incubated with purified HopZ3-His, washed and resolved by SDS-PAGE. Immunoblotting showed interactions between these proteins and HopZ3. (B-D) HopZ3 interacts with all three tomato RIN4s. Immobilized MBP-tagged SIRIN4-1 (B) or GST-tagged SIRIN4-2 (C) pulled down HopZ3-His and immobilized HopZ3-GST pulled down SIRIN4-3-His (D). (E) GST-tagged HopZ3 was pulled down using immobilized AvrPto1_{PSy}-His, showing their interaction. (F) HopZ3-GST was pulled down by immobilized AvrPtoB_{PSy}-His. Dashed lines in (A-C) separate input from pull down in the same blot and in (D, F) they mark protein weight standards.

HopZ3 and AvrPto1_{Psy} displayed similar protein–protein interaction profiles. AvrPto1_{Psy} directly interacted with the same tomato kinases and SIRIN4s as HopZ3 in at least one of the assays (Table 1 and S1-S3 Figs), which suggests these proteins are common targets for both effectors. As expected, recombinant AvrPto1_{Psy} could directly bind to PTO *in vitro* (Table 1 and S3A Fig), similarly to what was shown for AvrPto_{Pto} [17]. We also detected a weak signal using BIFC in *N. benthamiana*, suggesting *in planta* complex formation of AvrPto1_{Psy} and PTO (Table 1 and S2 Fig). However, AvrPto1_{Psy} did not show interaction with FEN *in vitro* or *in planta* (Table 1 and S2 and S3B Figs). In addition to HopZ3, AvrPto1_{Psy} also interacted with AvrPtoB_{Psy} in yeast two-hybrid and *in vitro* pull-down assays (Table 1 and S1 and S3F Figs). Many of HopZ3 interacting proteins interacted with each other (S1 and S2 Figs). These data show that HopZ3 directly targets the AvrPto-PTO defense pathway in tomato.

HopZ3 acetylates a subset of interacting proteins

Since HopZ3 has acetyltransferase activity [9], we tested whether several interacting proteins were its substrates *in vitro*, in reactions with ¹⁴C-acetyl-CoA and the cofactor inositol hexakisphosphate (IP6). Recombinant HopZ3, but not the catalytically inactive variant HopZ3_C300A, acetylated AvrPto1_{Psy} and its target PTO, SIRIPK, SIRIN4-1, SIRIN4-2 and SIRIN4-3 (Fig 3A and 3B). There was no detectable acetylation of FEN by HopZ3 (Fig 3B). Although AvrPtoB_{Psy} was capable of binding to HopZ3, it was not a good substrate for acetylation (Fig 3C). Despite diversity of substrates, HopZ3 activity is specific, as the enzyme does not acetylate interacting proteins MPK4 [9], FEN and AvrPtoB_{Psy} or non-interacting HopI_{Psy} [9].

Fig 3. HopZ3 acetylates SIRIN4-1,-2, -3, AvrPto1_{Psy}, PTO, SIRIPK but not FEN or AvrPtoB_{Psy}. Purified recombinant His-tagged SIRIN4-1, -2, -3, AvrPto1_{Psy}, AvrPtoB_{Psy} and GST-tagged PTO, FEN and SIRIPK proteins were incubated with His-tagged HopZ3 or HopZ3_C300A mutant (C/A) in the presence of IP₆ and ¹⁴C-acetyl-CoA for 2 h at 30°C. Samples were separated by SDS-PAGE and subjected to autoradiography for 14 days. (A) SIRIN4-1, -2, -3 and AvrPto1_{Psy} were acetylated by HopZ3. (B) PTO and SIRIPK were acetylated by HopZ3; however, FEN acetylation was not detected. (C) AvrPtoB_{Psy} was not significantly acetylated by HopZ3.

HopZ3 acetylates AvrPto1_{Psy} residues essential for interaction with PTO and decreases phosphorylation of residues involved in defense activation

To gain further insight into molecular mechanisms of immune suppression by HopZ3, we analyzed post-translational modifications of AvrPto1_{Psy} produced in *E. coli* and *N. benthamiana* by LC-MS/MS. By comparing acetylation sites found in *E. coli*-produced AvrPto1_{Psy} after *in vitro* acetylation reactions with ¹³C-acetyl-CoA, IP₆ and HopZ3 or HopZ3_C300A, we found that H125 and H130 were specifically acetylated by HopZ3 (S1 Table). These histidine residues were also specifically acetylated *in planta*, when AvrPto1_{Psy} and HopZ3 were co-expressed in *N. benthamiana*. Several other AvrPto1_{Psy} residues were acetylated *in vitro* and *in planta* to higher levels in the presence of HopZ3 compared to HopZ3_C300A (S1 Table and Figs 4 and S4). T91 and S94 in the

AvrPto1_{Psy} GINP Ω loop that is essential for interaction with PTO [15,17,29,30] were consistently found to be the most highly acetylated in several experiments (S1 Table). S46, which is also important for interaction with PTO [15,29,30] and the virulence function of AvrPto_{Pto} [31], was also acetylated by HopZ3. This residue is not in the binding interface, but likely stabilizes the protein fold [30].

Fig 4. HopZ3 acetylates multiple sites in AvrPto1_{Psy} and PTO important for their interaction and signaling. AvrPto1_{Psy} and PTO co-expressed with HopZ3 or HopZ3_C300A in *N. benthamiana* were analyzed using mass spectrometry for post translational modifications. (A–B) Models of the AvrPto1_{Psy} and PTO showing the modifications identified in the *in planta* experiment that are important for immune signaling. Models were developed using the iTASSER modeling server and algorithm. Major acetylation sites dependent on HopZ3 are shown in red, important phosphorylation sites in blue, sites either acetylated or phosphorylated in purple, known sites of interaction between AvrPto_{Pto} and PTO in yellow, acetylated interaction sites in orange and G2 myristoylation site in green. See also S1 and S2 Tables and S4 and S5 Figs. HopZ3 acetylates sites essential for interaction (orange) and decreases phosphorylation of residue(s) involved in signaling (blue box). (C) Model of HopZ3 acetylation sites in the crystal structure of PTO:AvrPto_{Pto} contact site [17]. AvrPto is shown in green with residues acetylated by HopZ3 in red, and PTO is shown in orange with sites acetylated by HopZ3 in blue. Modifications on either protein are in the known interaction area of the two proteins.

Many residues in AvrPto1_{Psy} produced in *E. coli* or in *N. benthamiana* were phosphorylated (S1 Table and Figs 4 and S4). Interestingly, S136 was very highly phosphorylated *in planta* (regardless of the presence of HopZ3), but it was not phosphorylated in the recombinant protein. This plant modification of AvrPto has not been reported previously; its functional significance is unknown and was not further explored. Since HopZ3 also targets serines and threonines, the same residues may also be phosphorylated. S147 and S149 of AvrPto1_{Psy} were phosphorylated *in vitro* and *in planta*, and HopZ3 acetylated a fraction of these residues as well. Importantly, in *N. benthamiana* expressing HopZ3, phosphorylation of S147 and/or S149 was significantly reduced (S1 Table). These residues were previously shown to be phosphorylated and contribute to the avirulence activity of AvrPto_{Pto} during interactions with resistant tomato [32] and *Nicotiana* sp. [33], as well as to virulence during susceptible tomato infection [32]. In our LC-MS/MS analysis, we also directly detected myristoylation of G2, a modification that enables membrane localization of AvrPto [32] (S1 Table and Figs 4 and S4).

Acetylation of residues in the AvrPto1_{Psy} Ω loop that interacts with PTO and decreased phosphorylation of residue(s) involved in signaling likely contribute to the mechanism by which HopZ3 reduces the immune response to AvrPto1_{Psy}.

Residues acetylated by HopZ3 are important for AvrPto1_{Psy} avirulence during tomato infection

Many residues acetylated by HopZ3 are important for the ability of AvrPto1_{Psy} to trigger a defense response in resistant tomato. For example, S94 and S147/S149 in AvrPto_{Pto} were shown to contribute to triggering PTO-mediated disease resistance and

were extensively studied, as discussed above. Although T91 in the GINP Ω loop was not found to affect interaction with PTO in any mutagenesis studies, a T91A variant that we constructed lost the ability to suppress the growth of *PsyB728a* Δ HopZ3 in PtoR tomato (Fig 5A) and was defective in the induction of cell death in *N. benthamiana* (S6 Fig). H125/H130 residues are on the opposite side of AvrPto1_{Psy} molecule from the Ω loop (Fig 4) and their substitutions did not disrupt *in vitro* binding to PTO (Fig 5B) or cell death induction in *N. benthamiana* (S6 Fig). Nevertheless, H125A/H130A substitutions reduced the ability of AvrPto1_{Psy} to suppress bacterial growth in resistant tomato (Fig 5A). Importantly, AvrPto1_{Psy} variants were expressed in *PsyB728a* to similar levels as wild-type AvrPto1_{Psy} (Fig 5C). Therefore, the residues acetylated by HopZ3 are important for the ability of AvrPto1_{Psy} to trigger a defense response in resistant tomato.

Fig 5. Effect of mutations of AvrPto1_{Psy} acetylation sites on *PsyB728a* growth in tomato. (A) AvrPto1_{Psy}_T91A and H125A/H130A mutants did not reduce *PsyB728a* growth in PTO-containing tomato in the absence of HopZ3. Plants were spray-inoculated with indicated strains at an OD₆₀₀=0.01. Epiphytic bacterial populations were quantified in leaf disc washes from eight different plants per strain four days after inoculation. Different letters indicate significant differences in growth as assessed by ANOVA with Tukey's test (P<0.05). Similar results were found in at least two other experiments (AvrPto1_{Psy}_H125A/H130A did not reduce the growth of *PsyB728a* Δ HopZ3 in three out of five experiments). Bars indicate standard errors. V, vector control; A1, AvrPto1_{Psy}; Z3, HopZ3; T91, AvrPto1_{Psy}_T91A; H125/H130 and HH, AvrPto1_{Psy}_H125A/H130A. (B) H125A/H130A mutation of AvrPto1_{Psy} did not affect its binding to PTO. AvrPto1_{Psy}-

GST, AvrPto1_{Psy}_H125A/H130A-GST and PTO-MBP were expressed in *E. coli*. Purified soluble AvrPto1_{Psy} or H125A/H130A mutant was pulled down with immobilized PTO-MBP or, alternatively, soluble PTO was pulled down with immobilized AvrPto1_{Psy}-GST or AvrPto1_{Psy}_H125A/H130A-GST. Band intensities were quantified from seven experiments. (T-test, P=0.1). (C) AvrPto1 mutant variants were expressed to similar levels as AvrPto1_{Psy} in ΔAvrPto1 and ΔhopZ3ΔAvrPto1 *Psy*B728a grown in type III secretion-inducing conditions.

HopZ3 acetylates key sites in the activation loop and other residues important for the immune function of PTO and reduces their phosphorylation

We used an LC-MS/MS analysis of PTO to gain insight into what specific effect acetylation might have. By comparing acetylation sites found in the presence of HopZ3 and HopZ3_C300A after *in vitro* acetylation reactions with ¹³C-acetyl-CoA, we identified T204 in the P+1 activation loop/region of PTO as a specific HopZ3-mediated acetylation site (S2 Table and S5 Fig). T204 is a cognate of T257 in Arabidopsis RIPK, another member of this kinase family that we found to be acetylated by HopZ3 [9].

T204 and T199 were the major acetylation sites *in planta* in PTO immunoprecipitated from *N. benthamiana* that also expressed functional HopZ3 (S2 Table and Figs 4 and S5). Both of these residues in the P+1 loop are important for interaction with AvrPto [16,17,20,22]. In addition, the structurally proximal residue K123 was acetylated in PTO co-expressed with HopZ3 *in planta*. Moreover, phosphorylation of

S198/T199 (and T190) was reduced in the presence of HopZ3 compared to HopZ3_C300A (S2 Table and Figs 4 and S5). Since phosphorylation of S198 and T199 is necessary for immune signaling [17,22,23], this may be a part of the mechanism by which HopZ3 reduces the plant defense response to AvrPto1_{PSy}.

Acetylation of AvrPto1_{PSy} and PTO affect their binding

A key step in the activation of AvrPto_{PTO}-triggered immunity requires its binding to PTO [19]. We hypothesized that modification by HopZ3 may affect the AvrPto1_{PSy}-PTO interaction because HopZ3 targets several residues in the binding interface (Fig 4 and S1 and S2 Tables). Therefore, we assayed the impact of AvrPto1_{PSy} or PTO acetylation on their interaction by performing *in vitro* acetylation reactions with HopZ3 followed by binding experiments. We found that binding was reduced when either AvrPto1_{PSy} or PTO was acetylated (Fig 6). Thus, part of the HopZ3 mechanism of immune suppression involves inhibition of the formation of the AvrPto1_{PSy}-PTO complex through their modification.

Fig 6. Effect of acetylation on AvrPto1_{PSy}-PTO binding. (A) Acetylation of AvrPto1_{PSy} reduces its interaction with PTO. Beads with immobilized His-AvrPto1_{PSy} were incubated for 2 h with acetyl-CoA, IP₆ and HopZ3, HopZ3_C300A (C/A), or no HopZ3 (un, untreated). Beads were washed and then incubated with soluble unmodified PTO-GST for 1 h, and after washing and elution, proteins were resolved by SDS-PAGE and stained with Coomassie blue or silver. The last two lanes in gel images are from different gels run at the same time as the other lanes, and interaction was always quantified relative to

immobilized protein in the same lane. The mean with the standard error of relative band intensities from at least four experiments is shown, with binding after reaction with HopZ3 set to 1. Different letters indicate significant differences (ANOVA/Fisher's test $P < 0.05$). Bars indicate standard errors. (B) Acetylation of PTO reduces its binding to AvrPto1_{PSy}. Experiments with immobilized acetylated PTO-GST and free AvrPto1_{PSy}-His were done as in (A).

Amino acid substitutions in PTO and FEN alter their acetylation specificity

FEN has an asparagine (N202) at the cognate position to T204 in PTO. Conversion of T204 to N in PTO abolished the acetylation of the protein by HopZ3 *in vitro* (Fig 7A). Conversely, mutating N202 to T in FEN rendered it susceptible to acetylation by HopZ3 (Fig 7B). The same amino acid substitutions switched the signaling specificity of PTO and FEN in response to AvrPto_{Pto} as assessed by cell death induction in transient expression experiments in *N. benthamiana* [16]. The loss of *in vitro* acetylation of PTO_T204N by HopZ3 is consistent with our finding of only one *in vitro* acetylation site in PTO by LC-MS/MS (S2 Table).

Fig 7. Substitutions in the P+1 activation loop of PTO and FEN affect their acetylation by HopZ3 and their kinase activity. Purified PTO-GST and FEN variants were incubated with HopZ3-His or HopZ3_C300A (C/A) mutant in the presence of IP₆ and ¹⁴C-acetyl-CoA (A–B) or γ^{32} P-ATP (C–D). Samples were separated by SDS-PAGE and subjected to autoradiography. (A) PTO but not a T204N P+1 activation loop variant

was acetylated by HopZ3. (B) The substitution of Asn202 to Thr in FEN conferred acetylation by HopZ3. (C–D) Kinase activity assay showing PTO and FEN autophosphorylation and transphosphorylation of HopZ3 and HopZ_C300A *in vitro*. Kinase variants with Thr (wild-type PTO and FEN_N202T) were more active than Arg or Asn variants.

Amino acid substitutions at position 204/202 greatly affected kinase activities of PTO and FEN, respectively. PTO and FEN variants with the T at 204/202 had higher kinase activity and showed more autophosphorylation than the N or R versions (Fig 7C and 7D; [17]). Together our data suggest that HopZ3 targets an essential residue in PTO that differentiates it from FEN in immune activation ability.

HopZ3 acetylates multiple sites in SIRIN4s and SIRIPK

We analyzed modifications of tomato RIN4s and RIPK acetylated *in vitro* by HopZ3 using ¹³C-acetyl-CoA and found many residues to be acetylated by HopZ3 (S3 and S4 Tables). We did not observe common modified sites among all three SIRIN4 paralogues and AtRIN4; however, these proteins are not highly conserved ([9], S7 Fig). The lack of conserved acetylations may also result from the intrinsically unstructured nature of RIN4s. We found one residue that is acetylated in tomato and Arabidopsis: S88 in SIRIN4-1/S79 in AtRIN4, respectively. This residue is conserved among RIN4s from many species [9,34]. The main regulatory phosphorylation sites corresponding to AtRIN4, T166 and S141 [6] were not acetylated by HopZ3 in tomato or Arabidopsis.

The major acetylation sites in AtRIPK [9] were acetylated by HopZ3 in the tomato orthologue. Similar to Arabidopsis, these sites could often be also phosphorylated

(S8 Fig). In particular, SIRIPK K120 (K122 in AtRIPK) in the ATP binding site, S219 (S221 in At) near the ATP binding site, SIRIPK S249/T250 (At S251/T252) in the activation loop and T255/H256 (T257 in At) were specifically acetylated by HopZ3 in both species; in addition, the serines/threonines were phosphorylation sites. K122 and S251/T252 in AtRIPK are necessary for RIPK activity [9] and S251/T252 are uridylated by the *Xanthomonas* effector AvrAC leading to RIPK inhibition [35]. Moreover, SIRIPK S249/T250 (At S251/T252) correspond to PTO S198/T199, whose phosphorylation is important for immunity [17,22,23] and is decreased by HopZ3 (S2 Table). The highest acetylation by HopZ3 was observed for SIRIPK T255, which corresponds to acetylated T257 in Arabidopsis RIPK and T204 in the PTO activation loop. Therefore, HopZ3 targets important residues conserved in SIRIPK, AtRIPK and PTO and directly acetylates SIRIPK residues necessary for kinase activity, acetylation of which may compete with phosphorylation.

PTO, FEN and SIRIPK phosphorylate HopZ3 and SIRIN4s, and are differentially affected by HopZ3 acetylation

We tested whether kinases from the RIPK family that interact with HopZ3 can phosphorylate HopZ3 and its putative targets, SIRIN4s. Indeed, PTO, FEN and SIRIPK phosphorylated HopZ3 and SIRIN4s *in vitro* (Figs 6C, 6D, S9 and 8).

Fig 8. Differential effects of HopZ3 acetylation on kinase activity of RIPK, PTO and FEN. GST fusions of SIRIPK (A–C) and FEN (D) or PTO-His (E–F) were incubated with or without acetyl-CoA, IP₆ and HopZ3 or HopZ3_C300A (C/A) for 2 h at RT and

then washed with PBS buffer. After acetylation, the kinase activity of SIRIPK, FEN or PTO was initiated by adding $\gamma^{32}\text{P}$ -ATP and MgSO_4 for 30 min. at RT. (A) Incubation of SIRIPK with HopZ3 in the absence of Acetyl-CoA did not affect the kinase activity of RIPK. (B) After incubation with acetyl-CoA and HopZ3, RIPK-mediated phosphorylation of itself and HopZ3 was reduced. (C) Phosphorylation of SIRIN4s was reduced after acetylation of SIRIPK by HopZ3. (D) Incubation with acetyl-CoA, IP_6 and HopZ3 did not affect the ability of FEN to phosphorylate itself. (E–F) Acetylation of PTO by HopZ3 did not affect PTO activity.

Next, we performed acetylation reactions with HopZ3 or HopZ3_C300A followed by kinase reactions. This permitted us to test the effect of acetylation on kinase activities. Acetylation of SIRIPK greatly reduced its kinase activity and phosphorylation of SIRIN4s and HopZ3 (Fig 8A-C), similar to what we observed with Arabidopsis RIPK [9]. These results confirm that HopZ3 targets SIRIPK sites that are important for activity (S8 Fig). As expected, incubation of FEN with HopZ3 in the acetylation reaction did not affect the autophosphorylation activity of FEN (Fig 8D); however, HopZ3 phosphorylation was lower than HopZ3_C300A, possibly due to autoacetylation of HopZ3. We expected that PTO activity may be suppressed by acetylation because an R substitution at T204, the residue acetylated by HopZ3, reduced its activity (Fig 7C). However, PTO kinase activity was not strongly affected by acetylation (Fig 8E and 8F). These data show a complex network of reciprocal modifications of HopZ3 and its substrates and suggest that HopZ3 does not exert its immune-suppressing effect by direct inhibition of PTO kinase activity.

498

499 **Discussion**

500 In this study, we explored the hypothesis that the HopZ3-dependent mechanism of
501 suppressing effector immune induction is conserved in diverse plant species, even when
502 the effectors triggering defenses and components of the plant immune complexes are
503 different. In resistant tomato, phosphorylation plays a prominent role in immune
504 activation, with phosphorylated residues in effector and plant proteins promoting
505 signaling [17,22,23,32,33]. The PTO kinase binds to the AvrPto1_{PSy} effector, leading to the
506 robust PRF-dependent restriction of bacterial growth. This study points to several
507 mechanisms by which HopZ3 disrupts the PTO pathway, as outlined in the model in Fig
508 9. In one mechanism, acetylation of residues at the binding interface of AvrPto1_{PSy} (T91,
509 S94) and PTO (T199, T204) or other residues needed for binding (S46 in AvrPto1_{PSy}),
510 disrupt the AvrPto1_{PSy}–PTO interaction and subsequent immune responses. Acetylation
511 can also directly compete at other sites for phosphorylation events that promote
512 activity/signaling of the targets. For example, S147/S149 residues in AvrPto1_{PSy} and
513 T199 in PTO are acetylated *in planta*, and phosphorylation of these residues is decreased
514 in the presence of active HopZ3. An additional mechanism could be inactivation of
515 kinases by acetylation; HopZ3 may also inhibit the unknown plant kinase(s) that
516 phosphorylates AvrPto1_{PSy}. It is also possible that acetylated AvrPto1_{PSy} is a poor kinase
517 substrate. Although we did not observe *in vitro* suppression of PTO kinase activity by
518 acetylation, it might be affected *in planta*, where more residues in the activation domain
519 are acetylated.

520

Fig 9. Model of HopZ3 disruption of PTO-mediated signaling in tomato. PTO and PRF are in a multimeric complex under basal conditions [23]; it is unknown whether RIN4 and/or RIPK are in a complex. AvrPto and RIN4 were shown to be associated with the membrane, but it is not known where the interactions occur. Upon infection with *P. syringae* containing AvrPto1_{Psy} but not HopZ3, AvrPto1_{Psy} becomes phosphorylated and binds to a PTO molecule, inhibiting its activity. Another molecule of PTO can autophosphorylate and transphosphorylate PTO bound to AvrPto1_{Psy}. The AvrPto1_{Psy}-PTO interaction and phosphorylations cause PRF activation and initiation of effector-triggered immunity [23]. RIN4 interacts with AvrPto1_{Psy} and may be phosphorylated by RIPK and/or PTO during infection and contribute to signaling. In the presence of HopZ3, acetylation of AvrPto1_{Psy}, PTO, RIPK and RIN4 leads to reduced phosphorylation and suppression of AvrPto1_{Psy}-PTO complex formation, ultimately resulting in disruption of effector-triggered immunity.

In addition to acetylation at serine, lysine or threonine typically seen with YopJ family acetyltransferases, HopZ3 can also modify histidine [9]. Here we confirmed this unusual activity of HopZ3, as several histidines in AvrPto1_{Psy} and SIRIPK were acetylated. In AvrPto1_{Psy}, H125/H130 residues are targets of HopZ3 acetylation and are required for the immune-inducing activity of AvrPto1_{Psy} in tomato. A similar observation was made in AvrB3, where substitution of H221 mitigated defense activation [9]. Although AvrPto1_{Psy} histidine substitution did not alter protein stability or binding to PTO, these sites might facilitate other protein dynamics or binding to different immune components.

Residues corresponding to T204, S198 and T199 in PTO were also acetylated by HopZ3 in RIPK from Arabidopsis [9] and tomato, interfering with phosphorylation and decreasing RIPK activity. Both PTO and SIRIPK (like AtRIPK [9]) could phosphorylate HopZ3 and three tomato RIN4 homologues. SIRIN4-1 is involved in PTO-PRF immunity triggered by several effectors, including AvrPto_{Pto} and AvrPtoB_{Pto}, that lead to its degradation [24]. In Arabidopsis, both RIN4 degradation by AvrRpt2 and phosphorylation by RIPK triggered by AvrRpm1 and AvrB, induce immunity. This phosphorylation is prevented by HopZ3, which modifies Arabidopsis RIPK, RIN4, AvrRpm1 and AvrB3 [9]. In tomato, HopZ3 also modifies the aforementioned proteins and reduces SIRIPK activity *in vitro*, resulting in the reduced phosphorylation of SIRIN4s. The significance of the phosphorylation of SIRIN4s in tomato is unknown, but their perturbations may be guarded by R proteins and involved in immunity via a mechanism similar to that in Arabidopsis.

Tomato kinases and SIRIN4s targeted by HopZ3 also interact with AvrPto1_{Psy}. Moreover, AvrPto1_{Psy}, AvrPtoB_{Psy} and several other HopZ3 targets interact with each other. Many effectors target the same host hub proteins essential for immunity [36], including multiple kinases involved in defense [37]. Interestingly, in bean the epistatic relationship between AvrPto1_{Psy} and HopZ3 is reversed from that seen in tomato such that AvrPto1_{Psy} suppresses defenses induced by HopZ3 [38]. Epistatic interactions of the effector repertoire (effectome) are revealed in the context of the host immune repertoire (targetome) [39]. It is possible that bacterial effectors act as multi-effector anti-immune complexes, similar to plant immune complexes. Further research is needed to reveal the dynamics of these mixed plant–effector complexes. HopZ3 modification of multiple

components of host defense pathways and bacterial effectors themselves may provide ways to balance the suppression of immune responses in different plants while maintaining the virulence functions of effectors.

A survey of public databases suggests that HopZ3 homologues are not present in *P. syringae* pv. tomato strains sequenced to date. However, many *P. syringae* strains contain HopZ3 and we do not know if they can infect tomato. Pathogens constantly evolve, acquire (or lose) new effectors and this may enable infection of new plant species. It is plausible that tomato pathovars could acquire HopZ3 and overcome PTO/PRF-mediated disease resistance in the future, or a HopZ3-containing strain could become adapted to tomato. Epistatic interactions between effectors determine host range and effector loss and gain allow changes in host range.

Remarkably, some of HopZ3 immune modulations mirror those of other YopJ family acetyltransferases. Effectors in human and animal pathogens, such as YopJ in *Yersinia* sp., AvrA in *Salmonella* and VopA in *Vibrio*, acetylate residues in activation loops and ATP binding sites of kinases in MAPK and IKK pathways, blocking their phosphorylation [40]. Plant pathogen YopJ family effectors from *Pseudomonas*, *Ralstonia* and *Xanthomonas* are much more diverse and are known to have a large spectrum of unrelated substrates [40]. So far, HopZ3 is unique in its strategy to modify other bacterial effectors in addition to their plant targets to reduce immune responses. The ability to post-translationally modify its own effectors adds another layer to the bacterial arsenal, in addition to the acquisition of effectors suppressing PAMP- or effector-triggered defenses and the evolution of multiple effector alleles that can avoid recognition.

Material and methods

Plant growth and bacterial infection

Tomato (*Solanum lycopersicum*) plants had the Rio Grande-PtoR (76R) background that has *Pto/Prf* locus introgressed from resistant *S. pimpinellifolium*; *pto11* and *prf3* are lines with mutated, nonfunctional *Pto* and *Prf* genes, respectively [41]. Tomato and *Nicotiana benthamiana* plants were grown under standard greenhouse conditions (22–24°C and 16/8 h light/dark photoperiods). Bacterial infection with *P. syringae* pv. *syringae* strain *PsyB728a* (and derivatives thereof) was performed with 4-week-old plants. Tomato plants were sprayed with a bacterial suspension (O.D₆₀₀=0.01 with 0.02% Silwet in 10 mM MgSO₄) and covered with a dome without holes. Eight to twelve leaf discs from at least four infected plants were collected 3-5 days post inoculation. For total bacteria count, individual discs were homogenized in 200 µl of 10 mM MgSO₄ and for epiphytic bacteria count, discs were washed to detach surface bacteria by vortexing in 1 ml of 10 mM MgSO₄ [7,8]. Samples were serially diluted and plated on LB medium containing appropriate antibiotics. Bacterial growth experiments were performed at least three times. Results obtained with total and epiphytic bacteria counts were very similar and these experiments were used interchangeably. Transient transformation of *N. benthamiana* leaves using *Agrobacterium* was performed as previously described [7]. Bacterial strains are listed in S5 Table.

Plasmid construction

For Gateway cloning vectors, the open reading frame (ORF) of each gene was amplified without a stop codon using Pfu-DNA polymerase (Agilent Technologies) and the entire region was cloned into pDONR207 by Gateway BP reaction (Life Technologies) and then recombined by Gateway LR reaction (Life Technologies) into the destination vectors (pG005/pG006 for BiFC, pLaw vectors for yeast two-hybrid assay, pBAV226 for expression in *PsyB728a*). Point mutations were introduced by PCR using overlapping primers with mutated codons. The *E. coli* protein expression vectors used in this study (S6 Table) are not Gateway compatible. The ORFs were amplified using gene-specific primers with restriction enzyme sites at the 5'-end or 3'-ends. PCR products were digested with specific restriction enzymes and ligated into expression vectors. All constructs were verified by sequencing. Details of primers, vectors, bacterial and yeast strains are provided in S5-S7 Tables.

Effector deletion strains and complementation

Unmarked deletions of *AvrPto1* in *PsyB728a* and *PsyB728aΔHopZ3* [8] were created as described [8,9]. Briefly, regions upstream and downstream of *AvrPto1* were amplified with 5' and 3' primers (S7 Table) and linked together in pMTN1907 that has *SacB* cassette for negative selection. Colonies with integrated plasmid were selected on kanamycin, and subsequently deletion strains were selected on 10% sucrose. Deletion strains were complemented with effectors expressed from the *nptII* promoter in the low-copy pBAV226 plasmid as previously described [8]. Details of vectors and primers are provided in S6 and S7 Tables.

GenBank accession numbers

635 GenBank accession numbers of proteins used in this study: AvrPto1_{PSy}:
636 AAY39946; AvrPtoB_{PSy} (HopAB1_{PSy}): Q4ZMD6; PTO: AAB47423; FEN: AAB47424;
637 SIRIPK: AAK62821; AtRIPK: NP_178651; SIRIN4-1: XP_010326285; SIRIN4-2:
638 XP_004242410; SIRIN4-3: XP_004252989; AtRIN4: NP_189143.

639

640 **Yeast two-hybrid assay**

641 The yeast two-hybrid screen was a part of a large scale effector-plant immune
642 signaling protein interaction screen ([9],
643 https://charge.ucdavis.edu/charge_db/interaction/Y2H/Y2H_interaction.php), and
644 identified interactions were confirmed as previously described [9]. Briefly, the
645 corresponding cells of the bait and prey were mated as shown in S1 Fig. Mated yeast
646 strains (S5 Table) expressing the bait and prey constructs were grown on the selective
647 minimal SD media (SD-Leu/-Trp/-His supplemented with 2.0 mM 3-aminotriazole (3-AT
648 and SD-Leu/-Trp/+X-gal) for 4–6 days. Experiments were performed at least twice.

649

650 ***In vitro* pull-down assay**

651 *In vitro* pull-down assays were performed between purified recombinant GST-
652 tagged SIPTO, -SIFEN, -SIRIN4-2, -3 or SIRIN4-1-MBP and His-tagged HopZ3;
653 between His-tagged AvrPto1_{PSy} or AvrPtoB_{PSy} and GST-tagged HopZ3, -PTO, or -FEN or
654 PTO-MBP as described [9]. Mixed proteins were incubated at 4°C for 1–2 h. Protein
655 bound to the glutathione-sepharose beads (GE Healthcare or Promega), Ni-NTA agarose
656 (QIAGEN) or amylose beads (NE BioLabs) was washed three to four times, separated on
657 SDS-PAGE and stained with Coomassie blue or immunoblotted with anti-GST, anti-

658 MBP and anti-His antibodies, respectively. All experiments were performed at least
659 twice.

660 To assess a protein–protein interaction after acetylation by HopZ3, beads with
661 immobilized AvrPto1_{PSY}-His or PTO-GST were incubated with 1 mM Acetyl-CoA, 5 μM
662 IP₆ and 1 μg HopZ3 or HopZ3_C300A for 2 h at room temperature (RT), washed three
663 times, then the second interacting protein was added and pull down was performed as
664 described above. Relative band intensities (interacting protein relative to immobilized
665 protein) were quantified from at least four independent experiments using Image Lab
666 software (Bio-Rad). To compare different experiments, interaction after acetylation with
667 HopZ3 was set to 1.

669 Immunoblotting

670 Proteins were resolved by 12% SDS-PAGE, transferred to a PVDF membrane and
671 probed with α-GST (Biolegend), α-His₆ (Clontech), α-MBP (NE BioLabs), α-GFP
672 (Clontech) and α-HA (Covance) antibodies followed by HRP-fused secondary antibodies
673 (Thermo Fisher Scientific). Blots were developed with chemiluminescent SuperSignal
674 Pico West solution (Thermo Fisher Scientific).

676 BIFC assay and confocal microscopy

677 For BIFC analysis, protein-coding sequences were cloned into expression
678 plasmids pG005 to create protein fused to the N-terminal half of YFP (protein:nYFP
679 fusions) and into pG006 to create protein fused to the C-terminal half of YFP
680 (protein:cYFP fusions), as previously described [9]. *N. benthamiana* leaves were co-

infiltrated with mixtures of Agrobacteria harboring indicated combinations of BIFC constructs and YFP fluorescence was imaged 2 days after agroinfiltration. A LSM710 confocal laser scanning microscope (Zeiss Microsystems) equipped with a 40X water-immersion objective was used to examine protein subcellular localization or protein–protein interaction in BIFC assays with *N. benthamiana* epidermal cells. GFP or YFP imaging was performed by excitation with 488 nm argon laser and emission at 496–544 nm for GFP and 494–573 nm for YFP. YFP fluorescence indicated interaction. Experiments were repeated two to three times.

***In vitro* acetylation**

Purified His-tagged HopZ3 or -HopZ3_C300A (0.5–1 µg) and 1–5 µg of potential substrates (GST tagged PTO, PTO_T204N, FEN and FEN_N202T; His-tagged SIRIN4-1, SIRIN4-2, SIRIN4-3, AvrPto1_{PSy} and AvrPtoB_{PSy}) were incubated with an acetylation reaction mixture containing 50 mM HEPES (pH 8.0), 10% glycerol, 5 µM IP₆ and 1–2 µl ¹⁴C-acetyl-coenzyme A (56 µCi/µM) (PerkinElmer Life Science) in a total volume of 20 µl as previously described [9]. The reactions were incubated for 2 h at RT and were terminated by the addition of SDS-PAGE loading buffer and boiling for 5 min. Proteins were separated by 12% or 15% SDS-PAGE, gels were dried on 3M paper and exposed to X-ray film for 7–14 days at -80°C. Experiments were performed two to three times.

***In vitro* kinase assay**

In vitro kinase assays were performed as previously described [9]. Briefly, 0.2, 0.4 and 0.6 µg of purified GST-tagged PTO or -FEN or 0.5 µg of purified GST-tagged

704 SIRIPK were incubated with a buffer containing 100 mM Tris 6.8, 10 mM MgCl₂, 10
705 mM MnCl₂, 10 μM ATP and 1 μl of γ-³²P-ATP and adding 2 μg of His-tagged SIRIN4-1
706 or HopZ3 at RT for 60 min. The reaction was stopped by adding 5x Laemmli buffer.
707 Proteins were separated by 12 or 15% SDS-PAGE, and signals were visualized by
708 autoradiography.

709 To determine kinase activity after acetylation by HopZ3, 1 μg of SIRIPK-GST,
710 FEN-GST or PTO-His were incubated with 1 mM Acetyl-CoA, 5 μM IP₆ and 1 μg of
711 His-Tagged HopZ3 or HopZ3_C300A for 2 h at RT and then washed with PBS. The
712 kinase activity of SIRIPK, FEN or PTO was initiated by adding ATP, γ-³²P-ATP and
713 MgSO₄ with or without SIRIN4s and incubated for 30 min at RT. All experiments were
714 performed two to three times.

716 ***In vitro* PTM mapping**

717 For *in vitro* acetylation mapping, reactions were performed with ¹³C-acetyl-CoA
718 (Acetyl-1,2-¹³C coenzyme A lithium salt, Sigma-Aldrich) to differentiate between
719 background ¹²C-acetylation that occurred in *E. coli* during the synthesis of recombinant
720 protein and HopZ3-mediated acetylation *in vitro*. Substrates were mixed with either
721 HopZ3 or the catalytically inactive HopZ3_C300A to distinguish any background
722 acetylation that could be chemically caused by the presence of ¹³C-acetyl-CoA. Briefly, 1
723 μg of purified His-tagged HopZ3 or HopZ3_C300A were mixed with 3 μg bead-bound
724 substrate to which the acetylation reaction cocktail (50 mM HEPES (pH 8.0), 10%
725 glycerol, 5 μM IP₆ and 50 μM of ¹³C-acetyl-CoA (Sigma-Aldrich)) was added in a total
726 volume of 20 μl. Subsequently, beads were washed twice with washing buffer (50 mM

HEPES pH 8.0, 50 mM NaCl, 10% glycerol), boiled in Laemmli loading buffer and processed for LC-MS/MS analysis. Data from the mass spectrometry of treated samples were analyzed for the presence of ¹³C-acetylated peptides in the substrate (AvrPto1_{Psy}, PTO, SIRIN4s, SIRIPK).

Immunoprecipitation and *in planta* PTM mapping

For *in planta* acetylation mapping, Dex-AvrPto1_{Psy}-HA or Dex-PTO-HA were transiently co-expressed with Dex-HopZ3-GFP or Dex-HopZ3_C300A-GFP constructs in *N. benthamiana*. Plants were treated with 30 μM dexamethasone for 16 h to induce protein production. Proteins were extracted with lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP40, 0.5% sodium deoxycholate, phosphatase inhibitor (Thermo Fisher Scientific), 2 μM sodium butyrate (TOCRIS Bioscience) and 3 μM trichostatin A (Sigma-Aldrich). Clarified total protein lysate was incubated for 3 h with anti-HA magnetic beads (Medical and Biological Laboratories Co., LTD) at 4°C. After washing the beads three times with the lysis buffer, proteins were eluted by boiling with Laemmli loading buffer. Samples were analyzed by LC-MS/MS. PTM mapping of AvrPto1_{Psy} and PTO was repeated with independent experiments.

LC-MS/MS analysis

Trypsin digestion and HPLC were performed as described [9]. Mass spectrometry was performed at the Medical Genome Facility Proteomics Core at Mayo Clinic, Rochester, MN, US. Samples were analyzed via liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) on a Q-Exactive (Thermo Fisher Scientific)

mass spectrometer, using a 60,000 RP survey scan, m/z 375–1950, with lockmasses, followed by 15 HCD (higher energy collisional dissociation) CID (collision-induced dissociation) scans on only doubly and triply charged precursors between 375 and 1950 Da and ions selected for MS/MS were placed on an exclusion list for 60 seconds. Inclusion lists were applied to enhance the detection of acetylated or phosphorylated peptides from specific targets. Briefly, using in house software to process the FASTA sequence file for AvrPto1_{PSY}, PTO, tomato RIN4_1-3 and SIRIPK, we performed *in silico* trypsinization and modeled the following modifications: (formyl n-term, oxidation (M), acetyl (K, H, S, T), ¹³C heavy acetyl (K, H, S, T), phospho (S, T), myristoylation (N-terminal G)), calculated m/z for doubly and triply charged ions, and combined the results into a *.csv file that was applied to the QE instrumentation method to enhance selection of the PTM-bearing ions for fragmentation. The MS data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) with the dataset identifier PXD022953. Database searching of the 160610_Greenberg_db9 database (3412 entries) and protein identification and PTM quantification were performed as described in [9] and [42]. All acetylated, phosphorylated and myristoylated peptide spectra were manually validated [9]. The second *in planta* experiment was quantified by TIC (total ion current) using Scaffold [43]. PTMs above 5% are shown in S1-S5 Tables.

Structural modeling

To assess the relevance of the acetylated residues found by mass spectrometry, we modeled the structure of the HopZ3 substrates using the iTASSER (Iterative Threading

Assembly Refinement) structural prediction software as previously described [9]. The best possible model was selected based on confidence score (C-score) calculated based on the significance of threading to the template alignments and convergence to the parameters of the structural assembly simulations. Model visualizations were generated using PyMOL software. PTM and interaction sites were labeled using the stick setting and coloring (Fig 4); however, the sites in the model are shown without PTMs.

Acknowledgments

We thank G.B. Martin (Boyce Thompson Institute) for tomato lines, G. Coaker (University of California, Davis) for SIRIN4 cDNAs and Elizabeth Georgian (University of California, Davis) for critical reading of the manuscript. We thank Misra Sengeldi for analyzing the prevalence of HopZ3 in sequenced *P. syringae* strains available in public databases.

References

1. Deslandes L, Rivas S. Catch me if you can: bacterial effectors and plant targets. *Trends in Plant Science*. 2012;17(11):644-55. doi: 10.1016/j.tplants.2012.06.011. PubMed PMID: 22796464.
2. Minton K. Plant immunity: Host mimicry of pathogen virulence targets. *Nature Reviews Immunology*. 2015;15(7):401. doi: 10.1038/nri3876. PubMed PMID: 26065587.
3. Liu J, Elmore JM, Lin ZJ, Coaker G. A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell Host & Microbe*. 2011;9(2):137-46. doi: 10.1016/j.chom.2011.01.010. PubMed PMID: 21320696.
4. Mackey D, Holt BF, 3rd, Wiig A, Dangl JL. RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell*. 2002;108(6):743-54. doi: 10.1016/s0092-8674(02)00661-x. PubMed PMID: 11955429.
5. Mackey D, Belkhadir Y, Alonso JM, Ecker JR, Dangl JL. *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*. 2003;112(3):379-89. doi: 10.1016/s0092-8674(03)00040-0. PubMed PMID: 12581527.
6. Chung EH, El-Kasmi F, He Y, Loehr A, Dangl JL. A plant phosphoswitch platform repeatedly targeted by type III effector proteins regulates the output of both tiers of plant immune receptors. *Cell Host & Microbe*. 2014;16(4):484-94. doi: 10.1016/j.chom.2014.09.004. PubMed PMID: 25299334.

809 7. Lee J, Teitzel GM, Munkvold K, del Pozo O, Martin GB, Michelmore RW, et al.
810 Type III secretion and effectors shape the survival and growth pattern of *Pseudomonas*
811 *syringae* on leaf surfaces. *Plant Physiology*. 2012;158(4):1803-18. doi:
812 10.1104/pp.111.190686. PubMed PMID: 22319072.

813 8. Vinatzer BA, Teitzel GM, Lee MW, Jelenska J, Hotton S, Fairfax K, et al. The
814 type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in
815 survival and disease on host and non-host plants. *Mol Microbiol*. 2006;62(1):26-44. doi:
816 10.1111/j.1365-2958.2006.05350.x. PubMed PMID: 16942603.

817 9. Lee J, Manning AJ, Wolfgeher D, Jelenska J, Cavanaugh KA, Xu H, et al.
818 Acetylation of an NB-LRR plant immune-effector complex suppresses immunity. *Cell*
819 *Rep*. 2015;13(8):1670-82. doi: 10.1016/j.celrep.2015.10.029. PubMed PMID: 26586425.

820 10. Lin NC, Martin GB. Pto- and Prf-mediated recognition of AvrPto and AvrPtoB
821 restricts the ability of diverse *Pseudomonas syringae* pathovars to infect tomato.
822 *Molecular Plant-Microbe Interactions: MPMI*. 2007;20(7):806-15. doi: 10.1094/MPMI-
823 20-7-0806. PubMed PMID: 17601168.

824 11. Kraus CM, Munkvold KR, Martin GB. Natural variation in tomato reveals
825 differences in the recognition of AvrPto and AvrPtoB effectors from *Pseudomonas*
826 *syringae*. *Molecular Plant*. 2016;9(5):639-49. doi: 10.1016/j.molp.2016.03.001. PubMed
827 PMID: 26993968.

828 12. Abramovitch RB, Kim YJ, Chen S, Dickman MB, Martin GB. *Pseudomonas* type
829 III effector AvrPtoB induces plant disease susceptibility by inhibition of host
830 programmed cell death. *The EMBO Journal*. 2003;22(1):60-9. doi:
831 10.1093/emboj/cdg006. PubMed PMID: 12505984.

- 832 13. Mathieu J, Schwizer S, Martin GB. Pto kinase binds two domains of AvrPtoB and
833 its proximity to the effector E3 ligase determines if it evades degradation and activates
834 plant immunity. *PLoS Pathogens*. 2014;10(7):e1004227. doi:
835 10.1371/journal.ppat.1004227. PubMed PMID: 25058029.
- 836 14. Rosebrock TR, Zeng L, Brady JJ, Abramovitch RB, Xiao F, Martin GB. A
837 bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity.
838 *Nature*. 2007;448(7151):370-4. doi: 10.1038/nature05966. PubMed PMID: 17637671.
- 839 15. Shan L, Thara VK, Martin GB, Zhou JM, Tang X. The pseudomonas AvrPto
840 protein is differentially recognized by tomato and tobacco and is localized to the plant
841 plasma membrane. *The Plant Cell*. 2000;12(12):2323-38. doi: 10.1105/tpc.12.12.2323.
842 PubMed PMID: 11148281.
- 843 16. Frederick RD, Thilmony RL, Sessa G, Martin GB. Recognition specificity for the
844 bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the
845 tomato Pto kinase. *Molecular Cell*. 1998;2(2):241-5. PubMed PMID: 9734361.
- 846 17. Xing W, Zou Y, Liu Q, Liu J, Luo X, Huang Q, et al. The structural basis for
847 activation of plant immunity by bacterial effector protein AvrPto. *Nature*.
848 2007;449(7159):243-7. doi: 10.1038/nature06109. PubMed PMID: 17694048.
- 849 18. Scofield SR, Tobias CM, Rathjen JP, Chang JH, Lavelle DT, Michelmore RW, et
850 al. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato.
851 *Science*. 1996;274(5295):2063-5. doi: 10.1126/science.274.5295.2063. PubMed PMID:
852 8953034.

- 853 19. Tang X, Frederick RD, Zhou J, Halterman DA, Jia Y, Martin GB. Initiation of
854 plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science*.
855 1996;274(5295):2060-3. doi: 10.1126/science.274.5295.2060. PubMed PMID: 8953033.
- 856 20. Rathjen JP, Chang JH, Staskawicz BJ, Michelmore RW. Constitutively active Pto
857 induces a Prf-dependent hypersensitive response in the absence of AvrPto. *The EMBO*
858 *Journal*. 1999;18(12):3232-40. doi: 10.1093/emboj/18.12.3232. PubMed PMID:
859 10369664.
- 860 21. Wu AJ, Andriotis VM, Durrant MC, Rathjen JP. A patch of surface-exposed
861 residues mediates negative regulation of immune signaling by tomato Pto kinase. *The*
862 *Plant Cell*. 2004;16(10):2809-21. doi: 10.1105/tpc.104.024141. PubMed PMID:
863 15367718.
- 864 22. Sessa G, D'Ascenzo M, Martin GB. Thr38 and Ser198 are Pto
865 autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response.
866 *The EMBO Journal*. 2000;19(10):2257-69. doi: 10.1093/emboj/19.10.2257. PubMed
867 PMID: 10811617.
- 868 23. Ntoukakis V, Balmuth AL, Mucyn TS, Gutierrez JR, Jones AM, Rathjen JP. The
869 tomato Prf complex is a molecular trap for bacterial effectors based on Pto
870 transphosphorylation. *PLoS Pathogens*. 2013;9(1):e1003123. doi:
871 10.1371/journal.ppat.1003123. PubMed PMID: 23382672.
- 872 24. Luo Y, Caldwell KS, Wroblewski T, Wright ME, Michelmore RW. Proteolysis of
873 a negative regulator of innate immunity is dependent on resistance genes in tomato and
874 *Nicotiana benthamiana* and induced by multiple bacterial effectors. *The Plant Cell*.
875 2009;21(8):2458-72. doi: 10.1105/tpc.107.056044. PubMed PMID: 19671880.

- 876 25. Conlan B, Stoll T, Gorman JJ, Saur I, Rathjen JP. Development of a rapid in
877 planta BioID system as a probe for plasma membrane-associated immunity proteins.
878 Front Plant Sci. 2018;9:1882. Epub 2019/01/09. doi: 10.3389/fpls.2018.01882. PubMed
879 PMID: 30619431.
- 880 26. Chien CF, Mathieu J, Hsu CH, Boyle P, Martin GB, Lin NC. Nonhost resistance
881 of tomato to the bean pathogen *Pseudomonas syringae* pv. *syringae* B728a is due to a
882 defective E3 ubiquitin ligase domain in AvrPtoBB728a. Molecular Plant-Microbe
883 Interactions: MPMI. 2013;26(4):387-97. doi: 10.1094/MPMI-08-12-0190-R. PubMed
884 PMID: 23252461.
- 885 27. Henry E, Toruno TY, Jauneau A, Deslandes L, Coaker G. Direct and indirect
886 visualization of bacterial effector delivery into diverse plant cell types during infection.
887 The Plant Cell. 2017;29(7):1555-70. Epub 2017/06/11. doi: 10.1105/tpc.17.00027.
888 PubMed PMID: 28600390.
- 889 28. Ronald PC, Salmeron JM, Carland FM, Staskawicz BJ. The cloned avirulence
890 gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance
891 gene. J Bacteriol. 1992;174(5):1604-11. Epub 1992/03/01. doi: 10.1128/jb.174.5.1604-
892 1611.1992. PubMed PMID: 1537802.
- 893 29. Chang JH, Tobias CM, Staskawicz BJ, Michelmore RW. Functional studies of the
894 bacterial avirulence protein AvrPto by mutational analysis. Molecular Plant-Microbe
895 Interactions: MPMI. 2001;14(4):451-9. doi: 10.1094/MPMI.2001.14.4.451. PubMed
896 PMID: 11310732.
- 897 30. Wulf J, Pascuzzi PE, Fahmy A, Martin GB, Nicholson LK. The solution structure
898 of type III effector protein AvrPto reveals conformational and dynamic features

899 important for plant pathogenesis. *Structure*. 2004;12(7):1257-68. doi:
 900 10.1016/j.str.2004.04.017. PubMed PMID: 15242602.

901 31. He P, Shan L, Lin NC, Martin GB, Kemmerling B, Nurnberger T, et al. Specific
 902 bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate
 903 immunity. *Cell*. 2006;125(3):563-75. Epub 2006/05/09. doi: 10.1016/j.cell.2006.02.047.
 904 PubMed PMID: 16678099.

905 32. Anderson JC, Pascuzzi PE, Xiao F, Sessa G, Martin GB. Host-mediated
 906 phosphorylation of type III effector AvrPto promotes *Pseudomonas* virulence and
 907 avirulence in tomato. *The Plant Cell*. 2006;18(2):502-14. doi: 10.1105/tpc.105.036590.
 908 PubMed PMID: 16399801.

909 33. Yeam I, Nguyen HP, Martin GB. Phosphorylation of the *Pseudomonas syringae*
 910 effector AvrPto is required for FLS2/BAK1-independent virulence activity and
 911 recognition by tobacco. *The Plant Journal*. 2010;61(1):16-24. doi: 10.1111/j.1365-
 912 313X.2009.04028.x. PubMed PMID: 19793077.

913 34. Toruno TY, Shen M, Coaker G, Mackey D. Regulated disorder: Posttranslational
 914 modifications control the RIN4 plant immune signaling hub. *Molecular Plant-Microbe*
 915 *Interactions: MPMI*. 2019;32(1):56-64. Epub 2018/11/13. doi: 10.1094/MPMI-07-18-
 916 0212-FI. PubMed PMID: 30418084.

917 35. Feng F, Yang F, Rong W, Wu X, Zhang J, Chen S, et al. A *Xanthomonas* uridine
 918 5'-monophosphate transferase inhibits plant immune kinases. *Nature*.
 919 2012;485(7396):114-8. Epub 2012/04/17. doi: 10.1038/nature10962. PubMed PMID:
 920 22504181.

921 36. Mukhtar MS, Carvunis AR, Dreze M, Epple P, Steinbrenner J, Moore J, et al.
922 Independently evolved virulence effectors converge onto hubs in a plant immune system
923 network. *Science*. 2011;333(6042):596-601. doi: 10.1126/science.1203659. PubMed
924 PMID: 21798943.

925 37. Brauer EK, Popescu GV, Singh DK, Calvino M, Gupta K, Gupta B, et al.
926 Integrative network-centric approach reveals signaling pathways associated with plant
927 resistance and susceptibility to *Pseudomonas syringae*. *PLoS Biology*.
928 2018;16(12):e2005956. doi: 10.1371/journal.pbio.2005956. PubMed PMID: 30540739.

929 38. Rufian JS, Lucia A, Rueda-Blanco J, Zumaquero A, Guevara CM, Ortiz-Martin I,
930 et al. Suppression of HopZ effector-triggered plant immunity in a natural pathosystem.
931 *Front Plant Sci*. 2018;9:977. Epub 2018/08/30. doi: 10.3389/fpls.2018.00977. PubMed
932 PMID: 30154802.

933 39. Arroyo-Velez N, Gonzalez-Fuente M, Peeters N, Lauber E, Noel LD. From
934 effectors to effectomes: Are functional studies of individual effectors enough to decipher
935 plant pathogen infectious strategies? *PLoS Pathogens*. 2020;16(12):e1009059. Epub
936 2020/12/04. doi: 10.1371/journal.ppat.1009059. PubMed PMID: 33270803.

937 40. Ma KW, Ma W. YopJ family effectors promote bacterial infection through a
938 unique acetyltransferase activity. *Microbiol Mol Biol Rev*. 2016;80(4):1011-27. Epub
939 2016/10/28. doi: 10.1128/MMBR.00032-16. PubMed PMID: 27784797.

940 41. Salmeron JM, Oldroyd GE, Rommens CM, Scofield SR, Kim HS, Lavelle DT, et
941 al. Tomato Prf is a member of the leucine-rich repeat class of plant disease resistance
942 genes and lies embedded within the Pto kinase gene cluster. *Cell*. 1996;86(1):123-33.
943 Epub 1996/07/12. doi: 10.1016/s0092-8674(00)80083-5. PubMed PMID: 8689679.

944 42. Manning AJ, Lee J, Wolfgeher DJ, Kron SJ, Greenberg JT. Simple strategies to
945 enhance discovery of acetylation post-translational modifications by quadrupole-orbitrap
946 LC-MS/MS. *Biochim Biophys Acta Proteins Proteom.* 2018;1866(2):224-9. Epub
947 2017/10/21. doi: 10.1016/j.bbapap.2017.10.006. PubMed PMID: 29050961.

948 43. Searle BC. Scaffold: a bioinformatic tool for validating MS/MS-based proteomic
949 studies. *Proteomics.* 2010;10(6):1265-9. Epub 2010/01/16. doi:
950 10.1002/pmic.200900437. PubMed PMID: 20077414.

951
952

Supporting information

S1 Fig. Yeast two-hybrid assay. Positive interactions are indicated by the growth on the selection medium without Trp, Leu and His (SD-WLH+5mM 3-AT) for the reporter gene HIS3 or by blue color on medium containing X-gal [9]. A schematic overview of a subset of tested combinations is represented in Table 1. SIRIN4-3_{trunc} was used as a negative control; it has a deletion of nucleotide 14 that caused a frameshift mutation and early stop. FEN as a bait caused auto-activation (false positive).

S2 Fig. Interaction between HopZ3, AvrPto1_{PSy} and their potential interactors in *planta*. Interactions of HopZ3, AvrPto1_{PSy}, AvrPtoB_{PSy}, PTO, FEN, SIRIPK, SIRIN4-1, -2 and -3 were tested by BiFC. YFP fluorescence was imaged by confocal microscopy in epidermal *N. benthamiana* cells co-infiltrated with mixtures of Agrobacteria harboring expression plasmids pG005 (protein:nYFP fusions) and pG006 (protein:cYFP fusions). Bar = 20 µm. Schematic overview of a subset of tested combinations is represented in Table 1: +, fluorescence detected; -, fluorescence not detected; weak, weak signal, as determined from images of several experimental samples.

S3 Fig. Pull downs with recombinant tagged proteins to assess interaction between AvrPto1_{PSy} and proteins in the PTO immune pathway. Empty Ni²⁺ resin or immobilized GST were used as negative controls for His- and GST- pull downs, respectively. Proteins were detected by Coomassie staining or immunoblotting. (A) Immobilized His-tagged AvrPto1_{PSy} pulled down purified PTO-MBP. (B) Immobilized AvrPto1_{PSy}-His was incubated with FEN-GST, showing no interaction. (C) Immobilized

His-tagged AvrPto1_{PSy} was incubated with SIRIN4-1-MBP showing weak interaction in one experiment and no interaction in two experiments. (D) Immobilized His-tagged AvrPto1_{PSy} was incubated with SIRIN4-2-GST showing weak interaction in two of three experiments. (E) Immobilized His-tagged AvrPto1_{PSy} was incubated with SIRIN4-3-GST. Interaction was not detected. (F) Immobilized GST-AvrPto1_{PSy} or GST was mixed with His-tagged AvrPtoB_{PSy}, showing that the two effectors interact.

S4 Fig. HopZ3 acetylates multiple sites in AvrPto1_{PSy} important for interaction with PTO and signaling. MS/MS spectra show PTM of AvrPto1_{PSy} expressed in *N. benthamiana* in the presence of HopZ3 or HopZ3_C300A. (A) Evidence of G2 myristoylation. (B) Acetylation of H125 and H130 in the presence of HopZ3. S136 was phosphorylated in both samples (HopZ3 and HopZ3_C300A). (C–E) Acetylation of S147 and S149 in the presence of HopZ3 (C) reduced phosphorylation of these residues. Phosphorylation was observed in the presence of HopZ3_C300A (D–E).

S5 Fig. HopZ3 acetylates key sites in the activation loop of PTO. PTMs were analyzed using mass spectrometry for PTO after *in vitro* acetylation reaction (A) or co-expressed with HopZ3 or HopZ3_C300A in *N. benthamiana* (B–E). (A–B) In both *in vitro* (A) and *in planta* (B) analyses T204, a key residue in the activation loop of the PTO kinase, was acetylated in the presence of HopZ3. (C) T199 acetylation in the presence of HopZ3. (D–E) Phosphorylation of S198 and T199 in the presence of HopZ3_C300A.

S6 Fig. Effect of mutations of AvrPto1_{PSy} acetylation sites on cell death induction in

***N. benthamiana*.** AvrPto1_{PSy}-GFP variants were transiently expressed in *N. benthamiana* infiltrated with Agrobacterium at OD=0.2 or 0.4 and sprayed with dexamethasone. (A) Only AvrPto1_{PSy}_T91A induced delayed cell death compared to wild-type AvrPto1_{PSy}. All variants were expressed to similar levels. A number of infiltrated areas with cell death per total number of samples is shown in the tables. (B) H125, H130 and double mutant of AvrPto1_{PSy} induced cell death in *N. benthamiana*.

S7 Fig. Alignment of tomato RIN4s (4_1, 4_2, 4_3) and Arabidopsis RIN4 (AT).

Modifications in SIRIN4s were determined *in vitro*, modifications in AtRIN4 are from [9] (*in vitro* and *in planta*). Residues acetylated by HopZ3 are bold and highlighted in yellow; phosphorylation sites are underlined; known phosphorylation sites important for signaling (S141, T166) in AtRIN4 [6] are highlighted blue; residues phosphorylated by RIPK in AtRIN4 (T21, S160, T166) [3] are circled in red. * (asterisk) - fully conserved residues, : (colon) - conservation between groups of strongly similar properties, . (period) - conservation between groups of weakly similar properties.

S8 Fig. HopZ3 acetylates SIRIPK residues important for activity. Modifications in

SIRIPK were determined *in vitro*, modifications in AtRIPK are from [9] (*in vitro* and *in planta*). Residues acetylated by HopZ3 are bold and highlighted in yellow; phosphorylation sites are underlined; known sites in AtRIPK important for activity (K122; S251/T252 which correspond to S198/T199 in PTO) [9] are circled in red; sites corresponding to T204 in PTO are circled in blue. * (asterisk) - fully conserved residues,

1021 : (colon) - conservation between groups of strongly similar properties, . (period) -
1022 conservation between groups of weakly similar properties.

1023

1024 **S9 Fig. Kinases in the PTO family can phosphorylate tomato RIN4s.** (A) PTO and
1025 FEN phosphorylate tomato SIRIN4-1. Kinase assays showing an increasing amount of
1026 PTO and FEN autophosphorylation and transphosphorylation of SIRIN4-1. (B-C) SIRIPK
1027 and FEN phosphorylate SIRIN4-3. The time course of the *in vitro* kinase reactions is
1028 shown. Purified SIRIPK (B) or FEN (C) were incubated in kinase buffer with or without
1029 SIRIN4-3 as a substrate. At indicated time points, aliquots of the reaction were taken out
1030 and separated by SDS-PAGE.

1031

1032 **S1 Table. AvrPto1_{PSy} PTMs *in vitro* and *in planta*.**

1033 PTMs were determined either *in vitro*, using purified recombinant AvrPto1_{PSy} after ¹³C-
1034 acetylation by HopZ3/HopZ3_C300A, or *in planta*, by co-expressing AvrPto1_{PSy} and
1035 HopZ3/HopZ3_C300A in *N. benthamiana*, followed by immunoprecipitation. Numbers
1036 indicate enrichment (fold change) of acetylation in the presence of HopZ3 vs.
1037 HopZ3_C300A. Red shading: significant (>50%) increase of acetylation with HopZ3.
1038 Blue shading: significant decrease of phosphorylation *in planta* in the presence of
1039 HopZ3. Residues known to be important for AvrPto signaling or interaction with PTO are
1040 in bold. + indicates phosphorylation found in a recombinant protein (*in vitro*) or *in*
1041 *planta*. Z3: acetylation found only in AvrPto1_{PSy} treated or co-expressed with HopZ3 and
1042 not HopZ3_C300A. Ac: acetylation; Phos: phosphorylation; Myr: myristoylation; exp:

1043 experiment. *Some spectra do not distinguish these 2 close residues. [#]*In planta* sites with
1044 acetylation above 25% in the presence of HopZ3.

1045

1046 **S2 Table. PTO PTMs *in vitro* and *in planta*.**

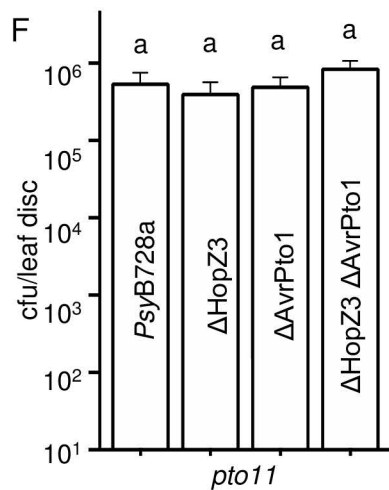
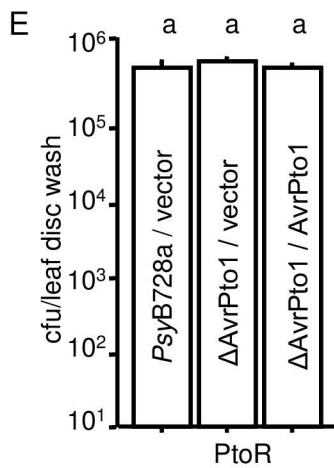
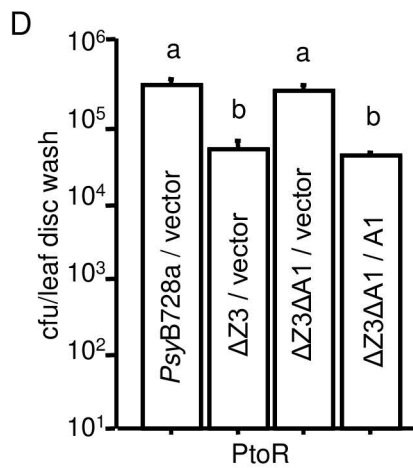
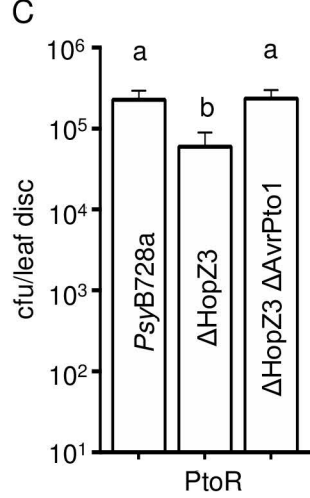
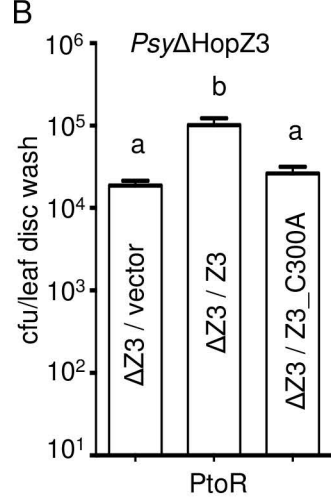
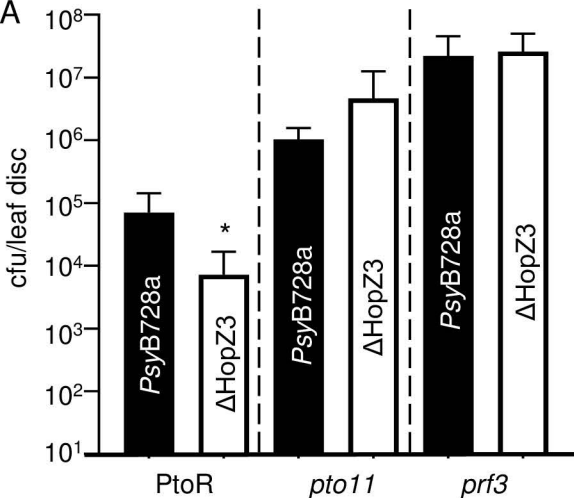
1047 PTMs were determined either *in vitro*, using purified recombinant PTO after ¹³C-
1048 acetylation by HopZ3/HopZ3_C300A, or *in planta*, by co-expressing PTO and
1049 HopZ3/HopZ3_C300A in *N. benthamiana*, followed by immunoprecipitation. Numbers
1050 indicate enrichment (fold change) of acetylation in the presence of HopZ3 vs.
1051 HopZ3_C300A. Red shading: significant (>50%) increase of acetylation with HopZ3.
1052 Blue shading: significant decrease of phosphorylation *in planta* in the presence of
1053 HopZ3. Residues important for PTO signaling or interaction with AvrPto are in bold. +
1054 indicates phosphorylation found in a recombinant protein (*in vitro*) or *in planta*. Z3:
1055 acetylation found only in PTO treated or co-expressed with HopZ3 and not
1056 HopZ33_C300A.; Ac: acetylation; Phos: phosphorylation; exp: experiment. *Some
1057 spectra do not distinguish these 2 close residues. [#]*In planta* sites with acetylation above
1058 25% in the presence of HopZ3.

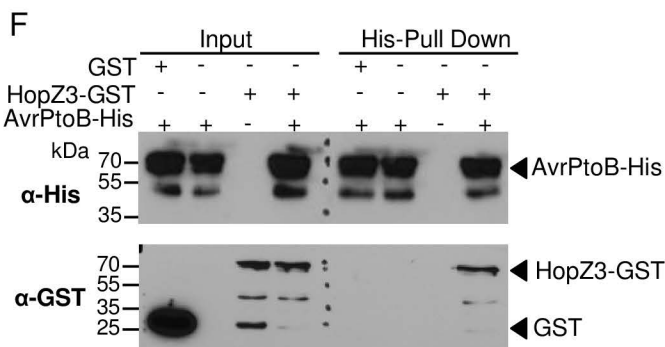
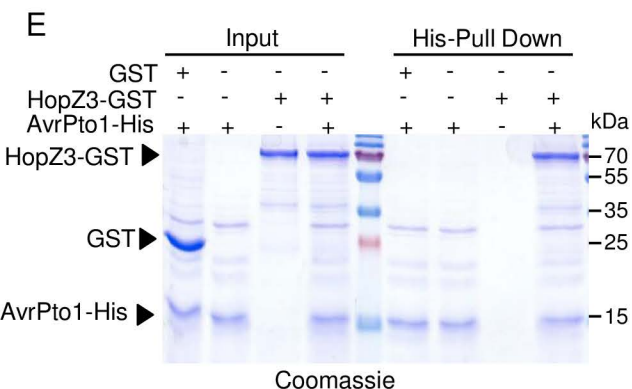
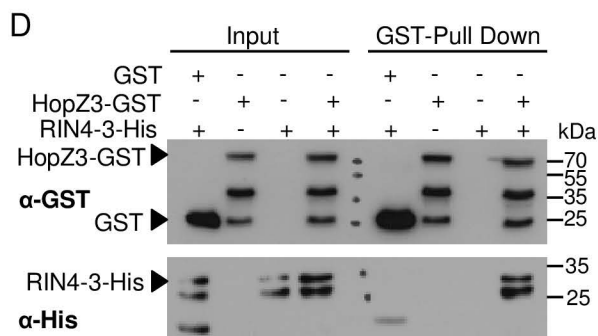
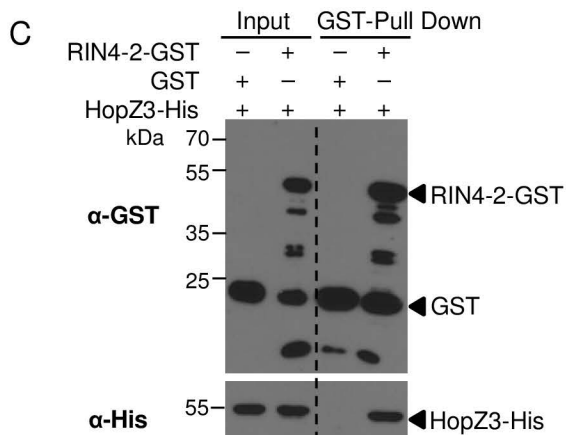
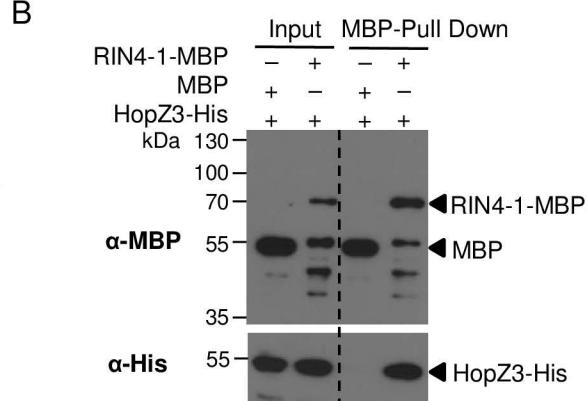
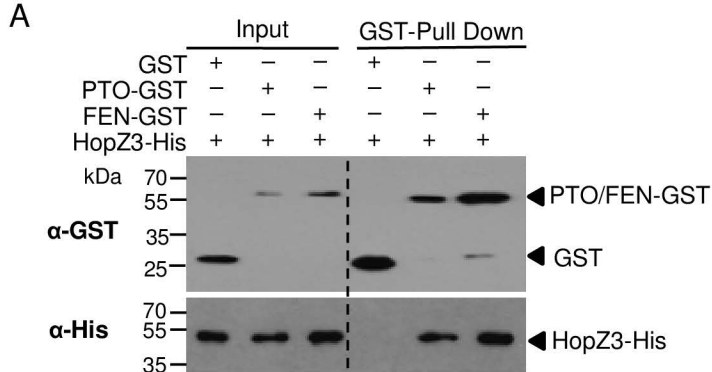
1059

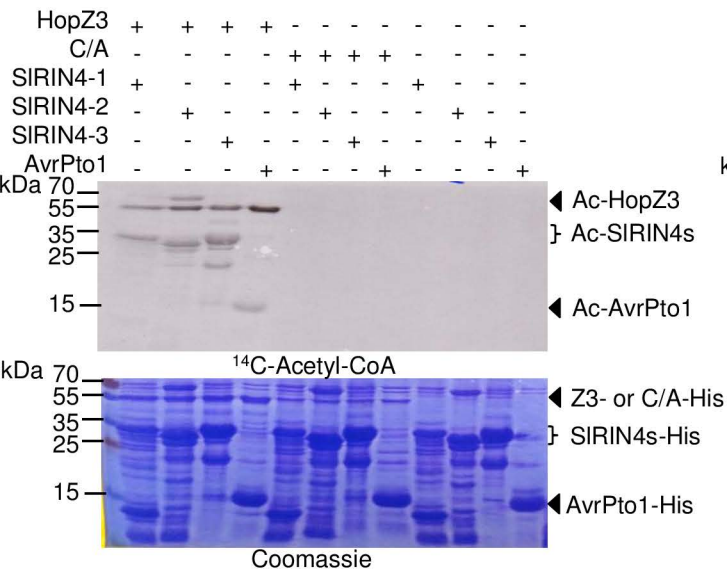
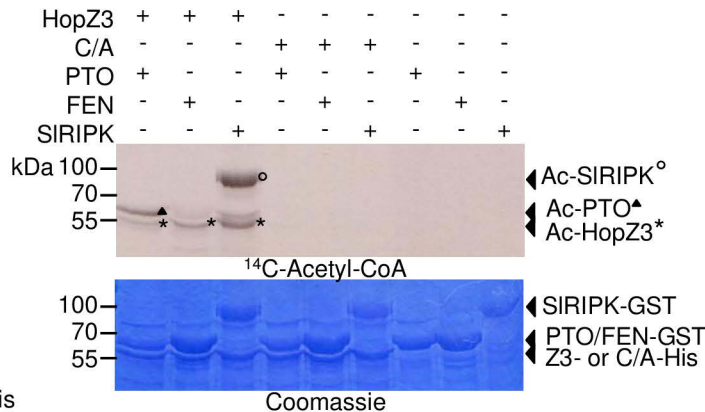
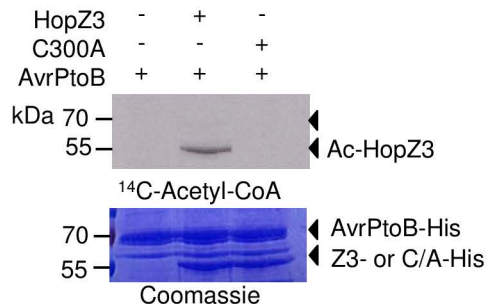
1060 **S3 Table. SIRIN4s PTMs *in vitro*.**

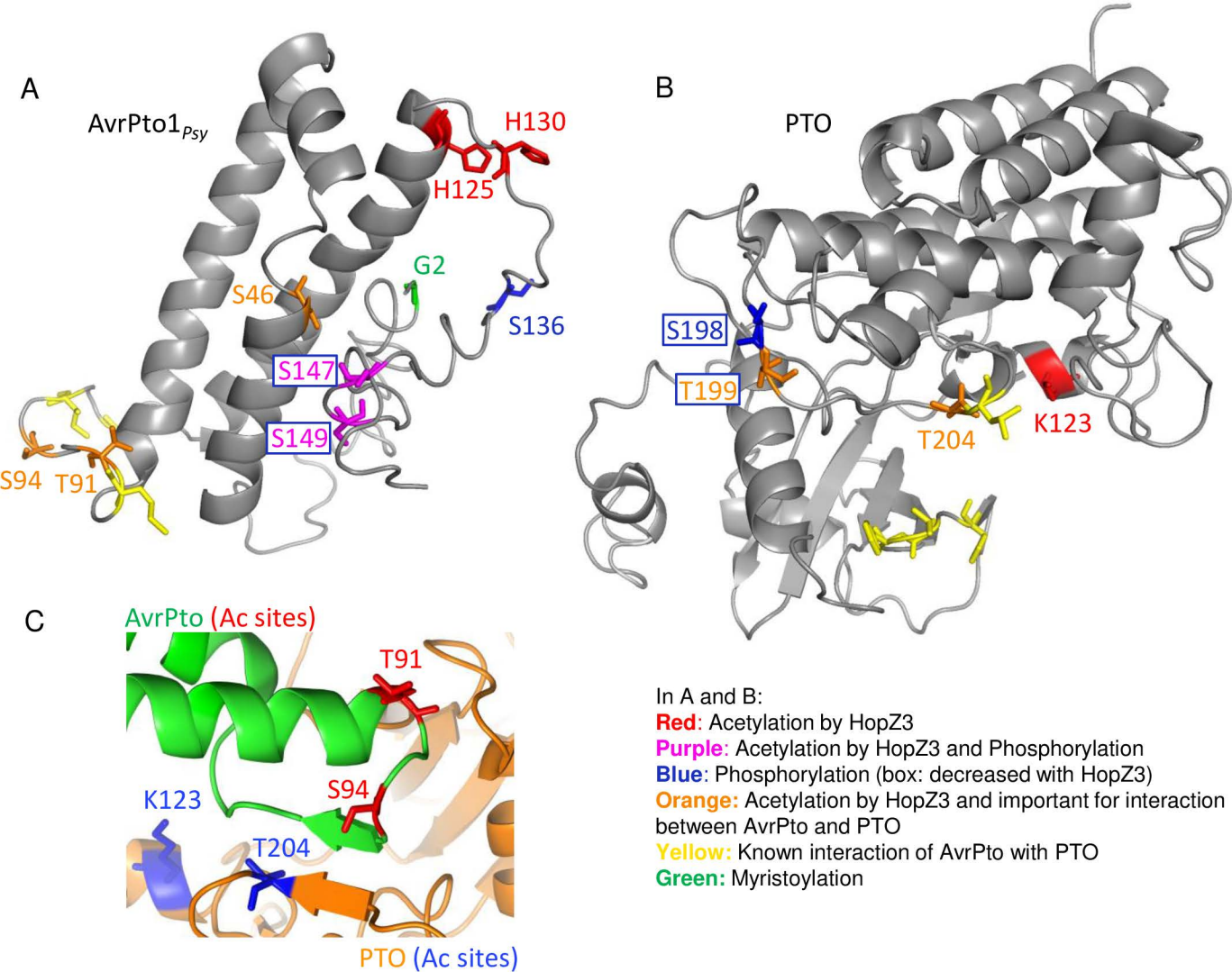
1061 PTMs were determined using purified recombinant SIRIN4s after *in vitro* ¹³C-acetylation
1062 by HopZ3/HopZ3_C300A. Numbers indicate enrichment (fold change) of ¹³C-acetylation
1063 in the presence of HopZ3 vs. HopZ3_C300A. Z3: acetylation found only in SIRIN4
1064 treated with HopZ3. Red shading: significant (>50%) increase of modification with

1065 HopZ3. + indicates phosphorylation found in a recombinant protein. Ac: acetylation;
 1066 Phos: phosphorylation.
 1067
 1068 **S4 Table. SIRIPK PTMs *in vitro*.**
 1069 PTMs were determined using purified recombinant SIRIPK after *in vitro* ¹³C-acetylation
 1070 by HopZ3/HopZ3_C300A. Numbers indicate enrichment (fold change) of ¹³C-acetylation
 1071 in the presence of HopZ3 vs. HopZ3_C300A. Z3: acetylation found only in SIRIPK
 1072 treated with HopZ3. Red shading: significant (>50%) increase of modification with
 1073 HopZ3. + indicates phosphorylation found in a recombinant protein. Ac: acetylation;
 1074 Phos: phosphorylation.
 1075
 1076 **S5 Table. Bacterial and yeast strains.**
 1077
 1078 **S6 Table. Plasmid vectors.**
 1079
 1080 **S7 Table. PCR primer sequences used in this study.** Mutated codons are underlined.
 1081



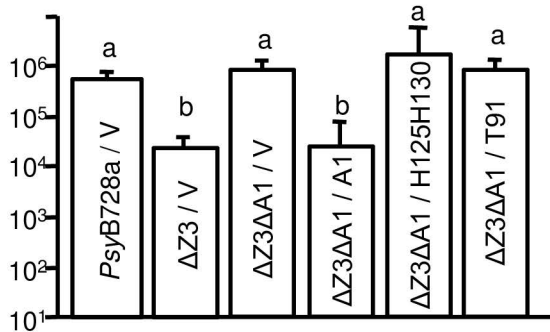


A**B****C**



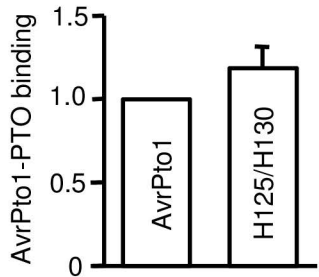
A

cfu/leaf disc wash



B

Binding to PTO



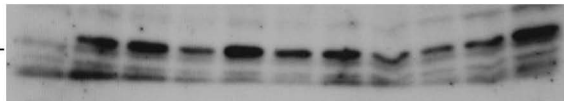
C

α-HA

strain ΔZ3ΔA1 ΔAvrPto1 ΔhopZ3ΔAvrPto1
 + V A1 HH T91 T91 A1 A1 HH HH T91 T91

kDa

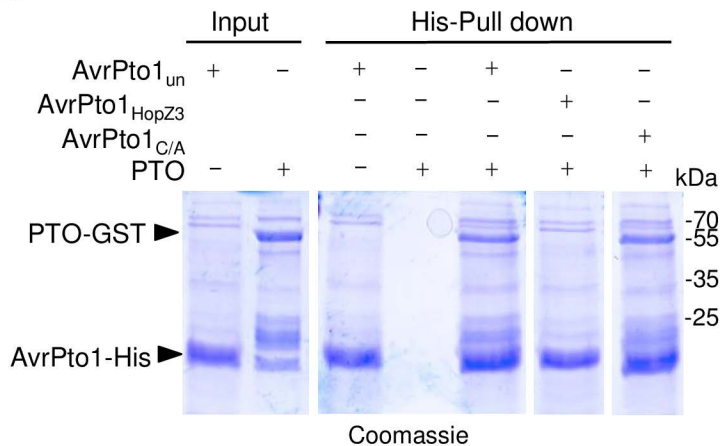
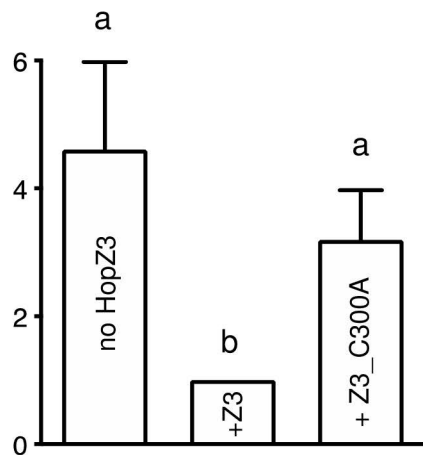
15



- AvrPto1-HA

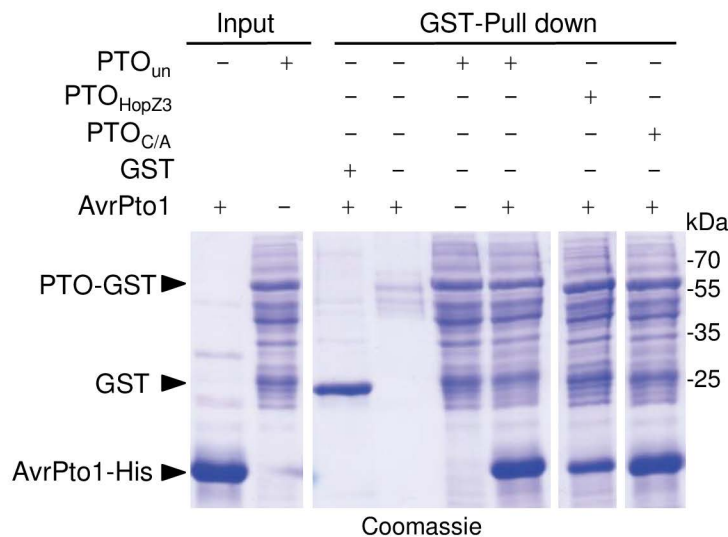
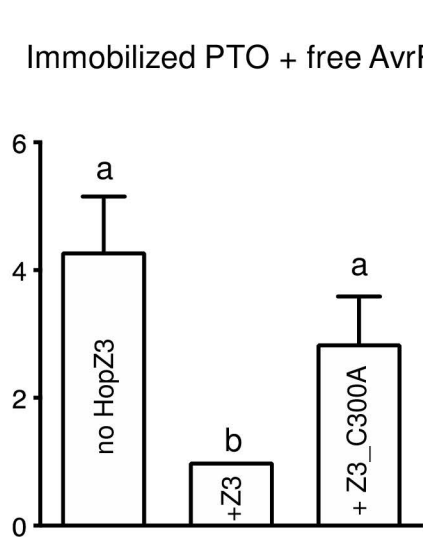
A Immobilized AvrPto1 + free PTO

PTO bound to AvrPto1



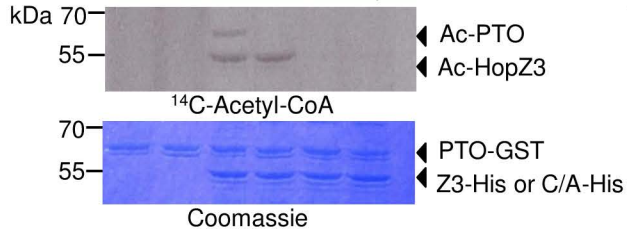
B Immobilized PTO + free AvrPto1

AvrPto1 bound to PTO



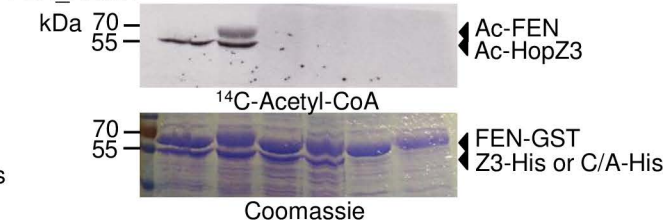
A

HopZ3	-	-	+	+	-	-
C/A	-	-	-	-	+	+
PTO	+	-	+	-	+	-
PTO_T204N	-	+	-	+	-	+



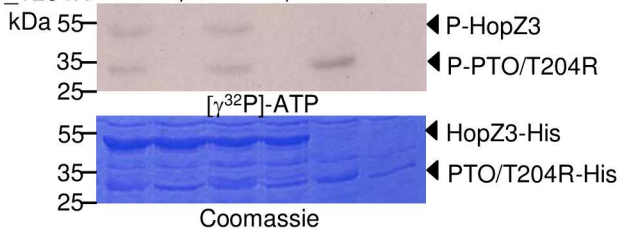
B

HopZ3	+	+	-	-	-	-
C/A	-	-	+	+	-	-
FEN	+	-	+	-	+	-
FEN_N202T	-	+	-	+	-	+



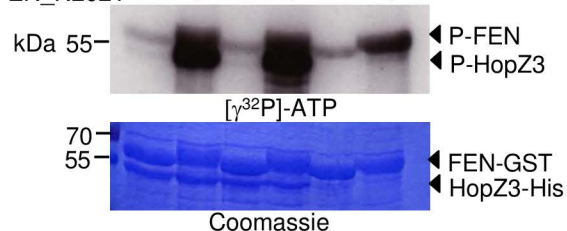
C

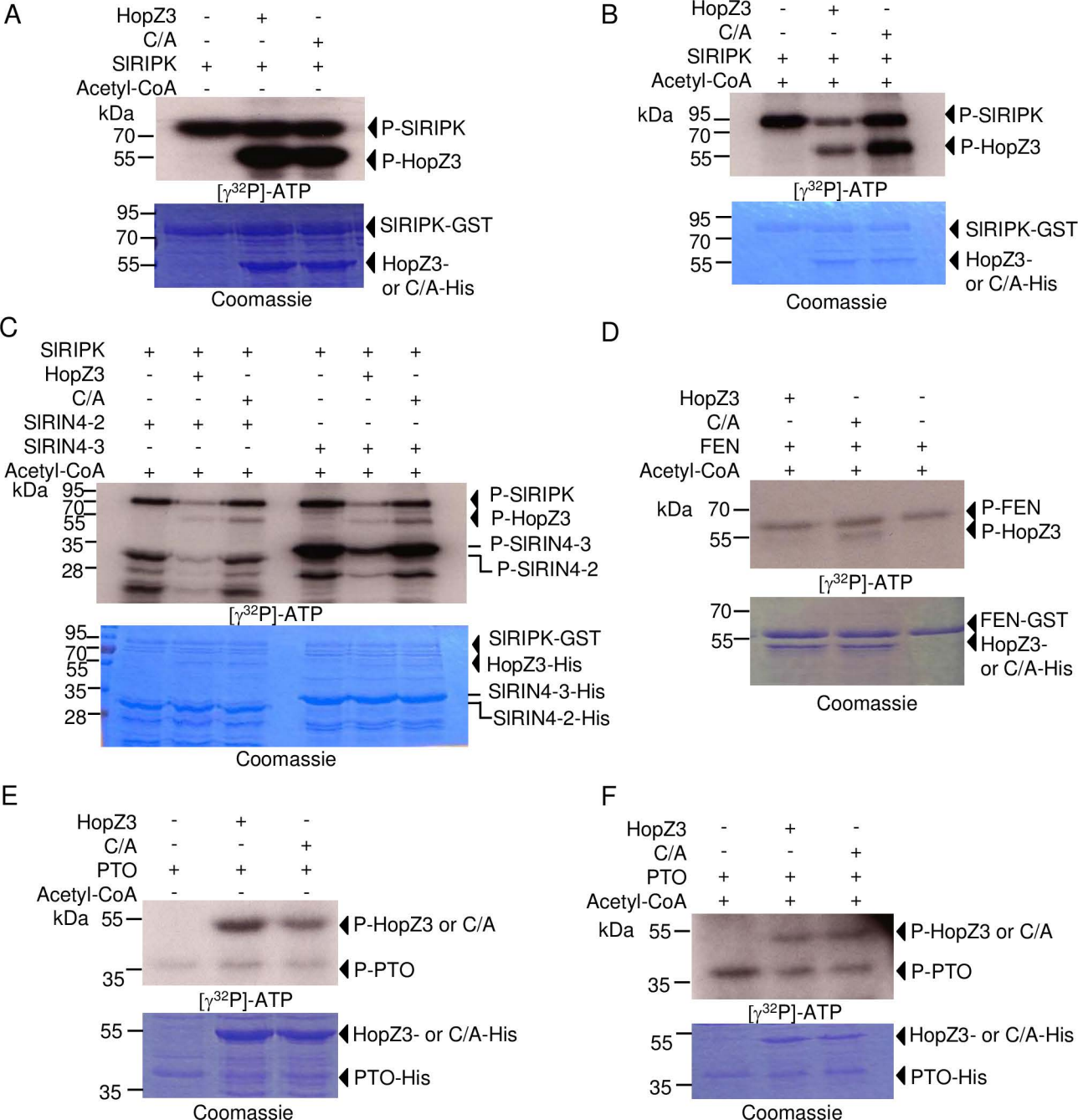
HopZ3	+	+	-	-	-	-
C/A	-	-	+	+	-	-
PTO	+	-	+	-	+	-
PTO_T204R	-	+	-	+	-	+



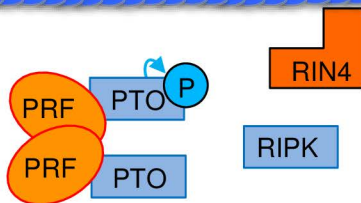
D

HopZ3	+	+	-	-	-	-
C/A	-	-	+	+	-	-
FEN	+	-	+	-	+	-
FEN_N202T	-	+	-	+	-	+





Tomato cell



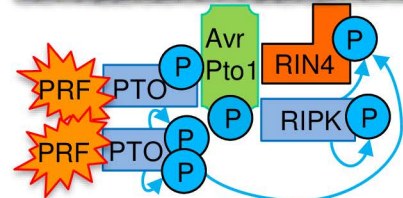
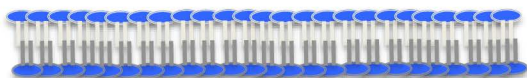
P. syringae
without HopZ3



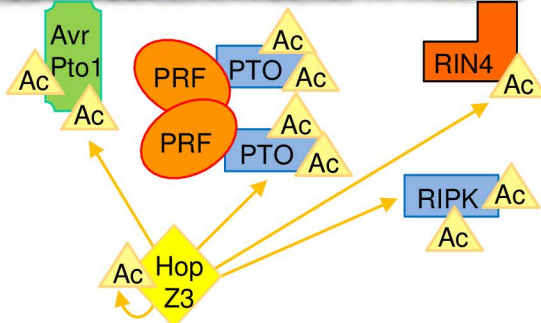
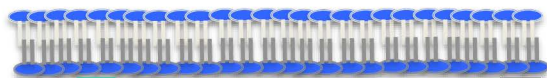
Infection



P. syringae
with HopZ3



Effector-triggered
immunity



Effector-triggered
immunity

	Bait	Prey	-WLH+3AT	-WL+X-gal	interaction
1.	HopZ3	PTO			-
2.	HopZ3	FEN			+ (weak)
3.	HopZ3	SIRIPK			-
4.	HopZ3	SIRIN4-1			-
5.	HopZ3	SIRIN4-2			-
6.	HopZ3	SIRIN4-3 _{trunc}			-
7.	PTO	HopZ3			-
8.	FEN	HopZ3			auto activation
9.	SIRIPK	HopZ3			+ (weak)
10.	SIRIN4-1	HopZ3			+
11.	SIRIN4-2	HopZ3			+
12.	SIRIN4-3 _{trunc}	HopZ3			-

	Bait	Prey	-WLH+3AT	-WL+X-gal	interaction
25.	PTO	PTO			-
26.	FEN	FEN			auto activation
27.	SIRIPK	SIRIPK			-
28.	SIRIN4-1	SIRIN4-1			+ (weak)
29.	SIRIN4-2	SIRIN4-2			-
30.	SIRIN4-3 _{trunc}	SIRIN4-3 _{trunc}			-
31.	PTO	SIRIN4-1			-
32.	PTO	SIRIN4-2			-
33.	PTO	SIRIN4-3 _{trunc}			-
34.	FEN	SIRIN4-1			auto activation
35.	FEN	SIRIN4-2			auto activation
36.	FEN	SIRIN4-3 _{trunc}			auto activation

	Bait	Prey	-WLH+3AT	-WL+X-gal	interaction
13.	AvrPto1	PTO			+ (weak)
14.	AvrPto1	FEN			+
15.	AvrPto1	SIRIPK			+
16.	AvrPto1	SIRIN4-1			-
17.	AvrPto1	SIRIN4-2			-
18.	AvrPto1	SIRIN4-3 _{trunc}			-
19.	PTO	AvrPto1			+
20.	FEN	AvrPto1			auto activation
21.	SIRIPK	AvrPto1			-
22.	SIRIN4-1	AvrPto1			+ (weak)
23.	SIRIN4-2	AvrPto1			+ (weak)
24.	SIRIN4-3 _{trunc}	AvrPto1			-

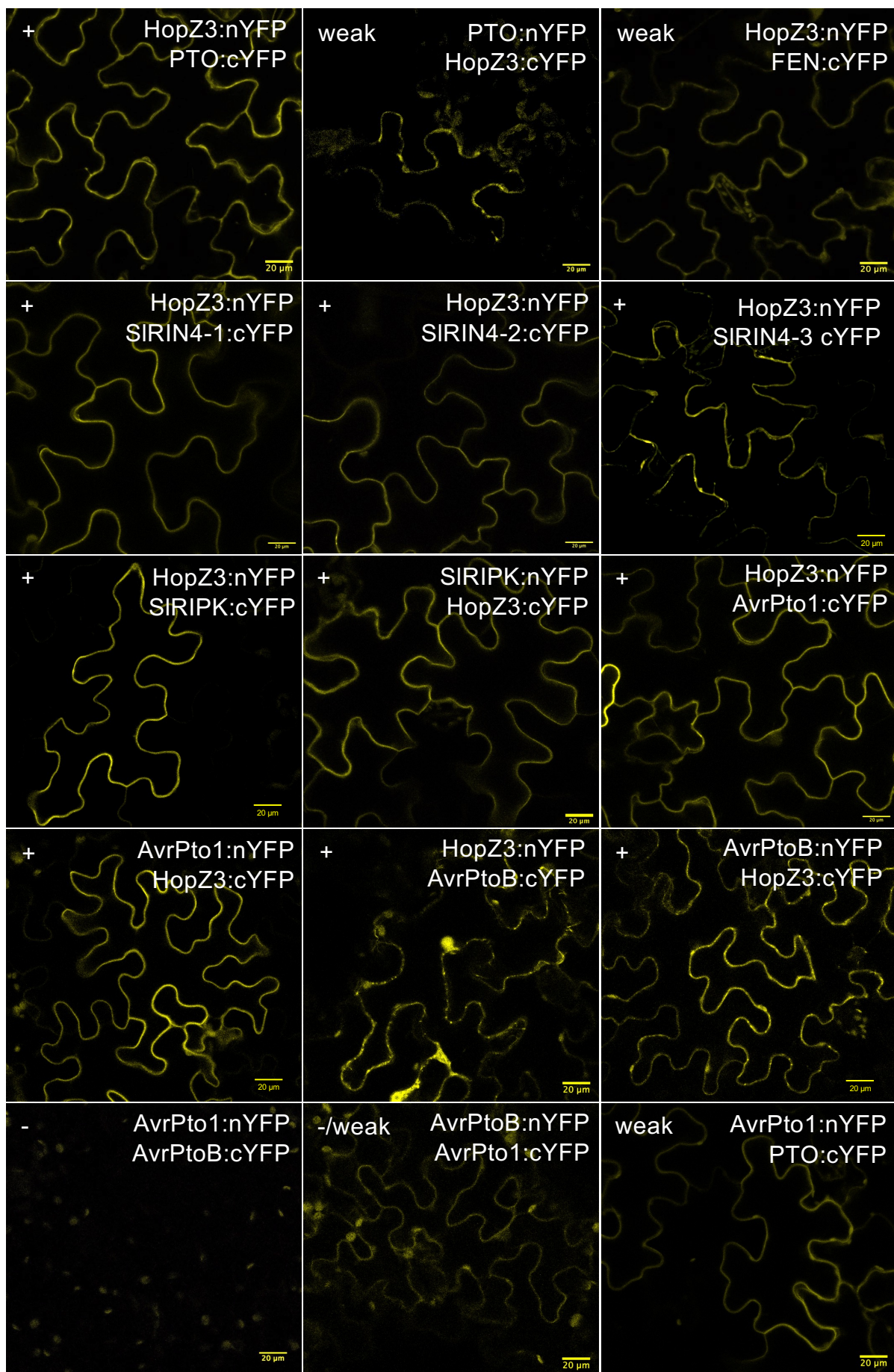
	Bait	Prey	-WLH+3AT	-WL+X-gal	interaction
37.	SIRIPK	SIRIN4-1			-
38.	SIRIPK	SIRIN4-2			-
39.	SIRIPK	SIRIN4-3 _{trunc}			-
40.	SIRIN4-1	PTO			-
41.	SIRIN4-2	PTO			+ (weak)
42.	SIRIN4-3 _{trunc}	PTO			-
43.	SIRIN4-1	FEN			+ (weak)
44.	SIRIN4-2	FEN			+
45.	SIRIN4-3 _{trunc}	FEN			-
46.	SIRIN4-1	SIRIPK			+ (weak)
47.	SIRIN4-2	SIRIPK			+
48.	SIRIN4-3 _{trunc}	SIRIPK			-

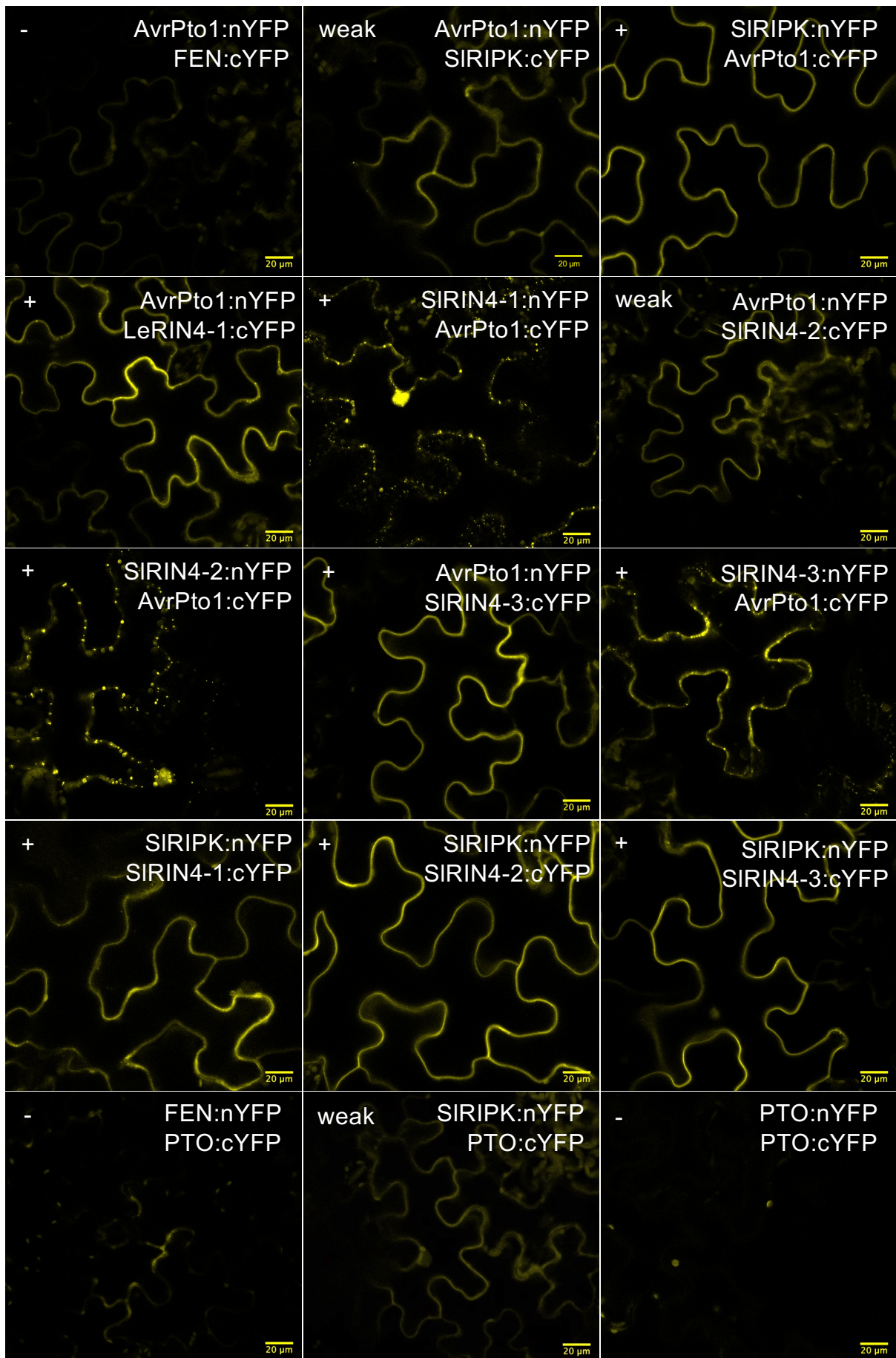
	Bait	Prey	-WLH+3AT	-WL+X-gal	interaction		Bait	Prey	-WLH+3AT	-WL+X-gal	interaction
49.	PTO	FEN			-	61.	HopZ3	AvrPto1			+
50.	PTO	SIRIPK			-	62.	HopZ3	AvrPtoB			+
51.	FEN	PTO			auto activation	63.	AvrPto1	HopZ3			+
52.	FEN	SIRIPK			auto activation	64.	AvrPtoB	HopZ3			+
53.	SIRIPK	PTO			-	65.	AvrPto1	AvrPtoB			+
54.	SIRIPK	FEN			+ (weak)	66.	AvrPtoB	AvrPto1			+
55.	SIRIN4-1	SIRIN4-2			-	67.	AvrPto1	AvrPto1			+
56.	SIRIN4-1	SIRIN4-3 _{trunc}			-	68.	AvrPtoB	AvrPtoB			+
57.	SIRIN4-2	SIRIN4-1			-						
58.	SIRIN4-2	SIRIN4-3 _{trunc}			-						
59.	SIRIN4-3 _{trunc}	SIRIN4-1			-						
60.	SIRIN4-3 _{trunc}	SIRIN4-2			-						

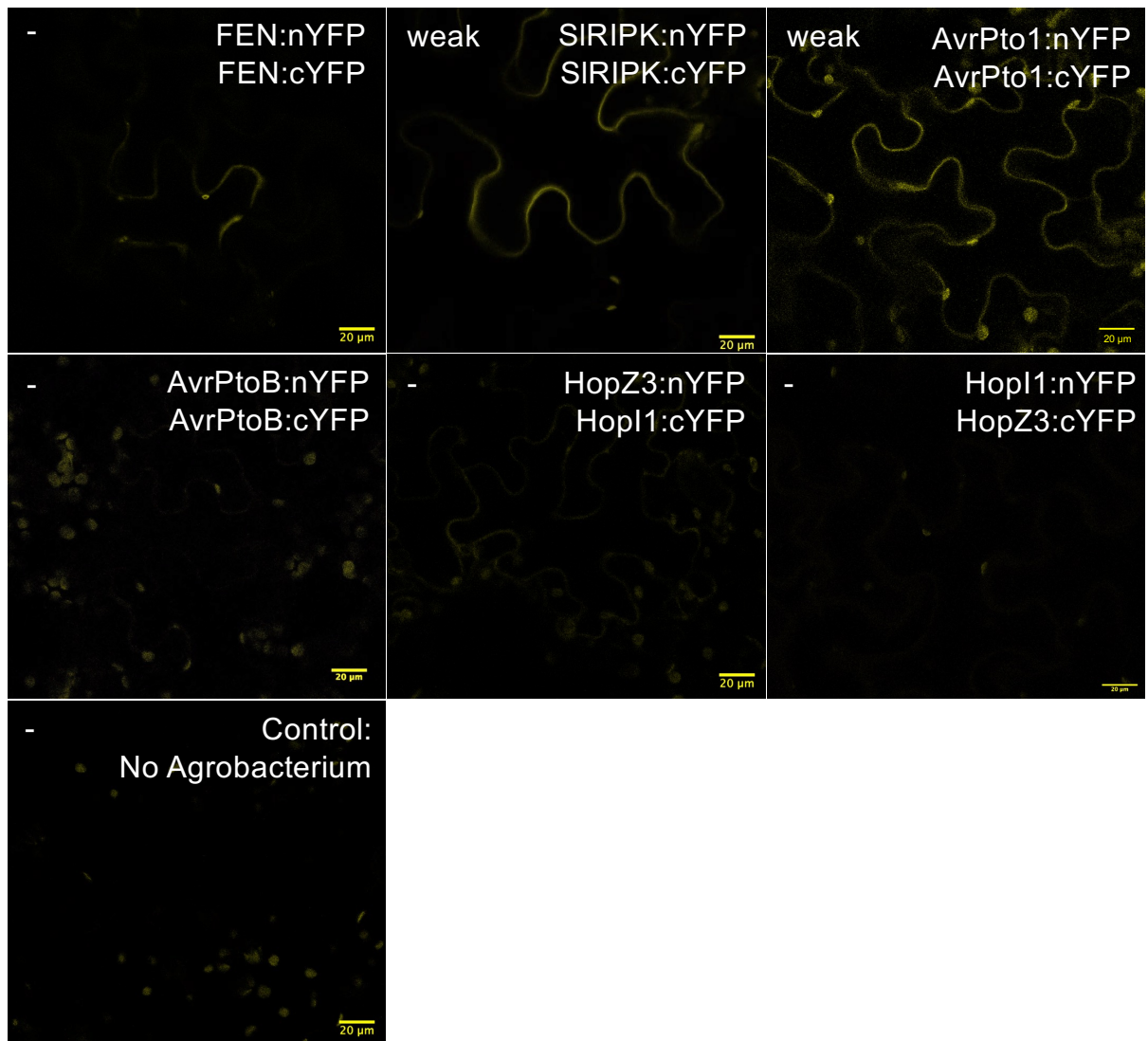
S1 Fig. Yeast two-hybrid assay. Positive interactions are indicated by the growth on the selection medium without Trp, Leu and His (SD-WLH+5mM 3-AT) for the reporter gene HIS3 or by blue color on medium containing X-gal [1]. A schematic overview of a subset of tested combinations is represented in Table 1. SIRIN4-3_{trunc} was used as a negative control, it has a deletion of nucleotide 14 that caused a frameshift mutation and early stop. FEN as a bait caused auto-activation (false positive).

References

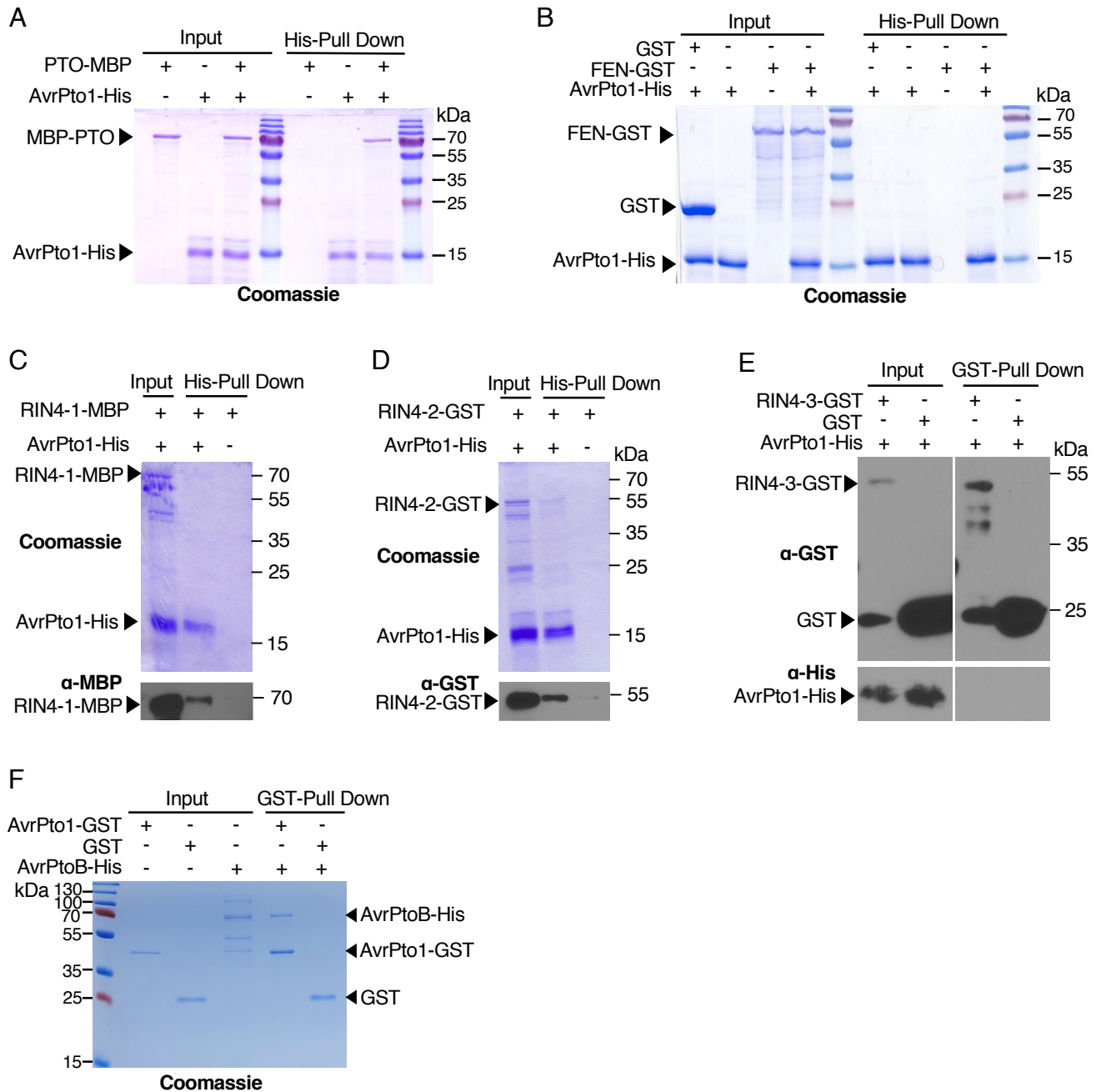
1. Lee J, Manning AJ, Wolfgeher D, Jelenska J, Cavanaugh KA, Xu H, et al. Acetylation of an NB-LRR plant immune-effector complex suppresses immunity. *Cell Rep.* 2015;13(8):1670-82. doi: 10.1016/j.celrep.2015.10.029. PubMed PMID: 26586425.





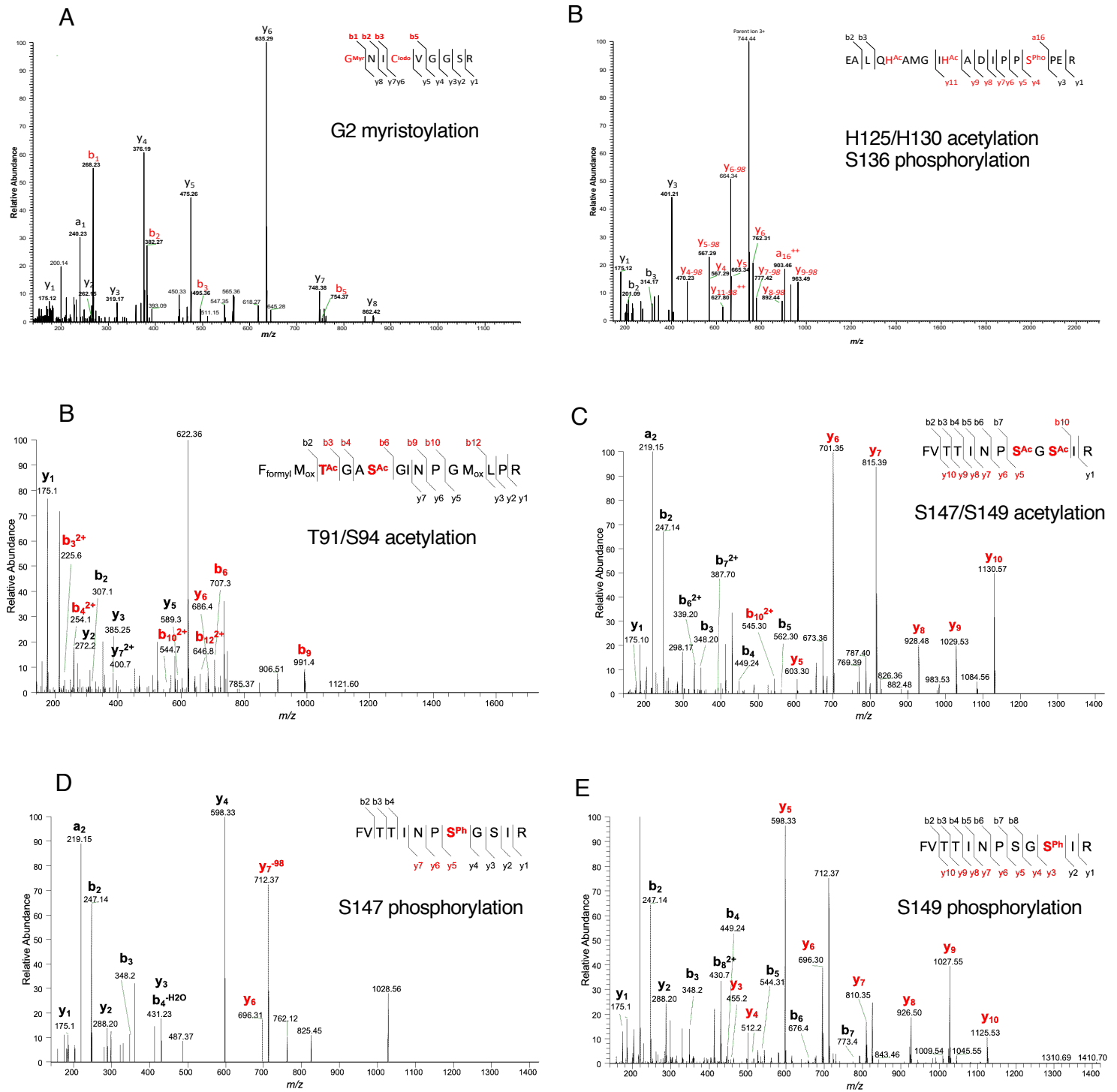


S2 Fig. Interaction between HopZ3, AvrPto1_{PSY} and their potential interactors in *planta*. Interactions of HopZ3, AvrPto1_{PSY}, AvrPtoB_{PSY}, PTO, FEN, SIRIPK, SIRIN4-1, -2 and -3 were tested by BiFC. YFP fluorescence was imaged by confocal microscopy in epidermal *N. benthamiana* cells co-infiltrated with mixtures of Agrobacteria harboring expression plasmids pG005 (protein:nYFP fusions) and pG006 (protein:cYFP fusions). Bar = 20 µm. Schematic overview of a subset of tested combinations is represented in Table 1. +, fluorescence detected; -, fluorescence not detected; weak, weak signal, as determined from images of several experimental samples.



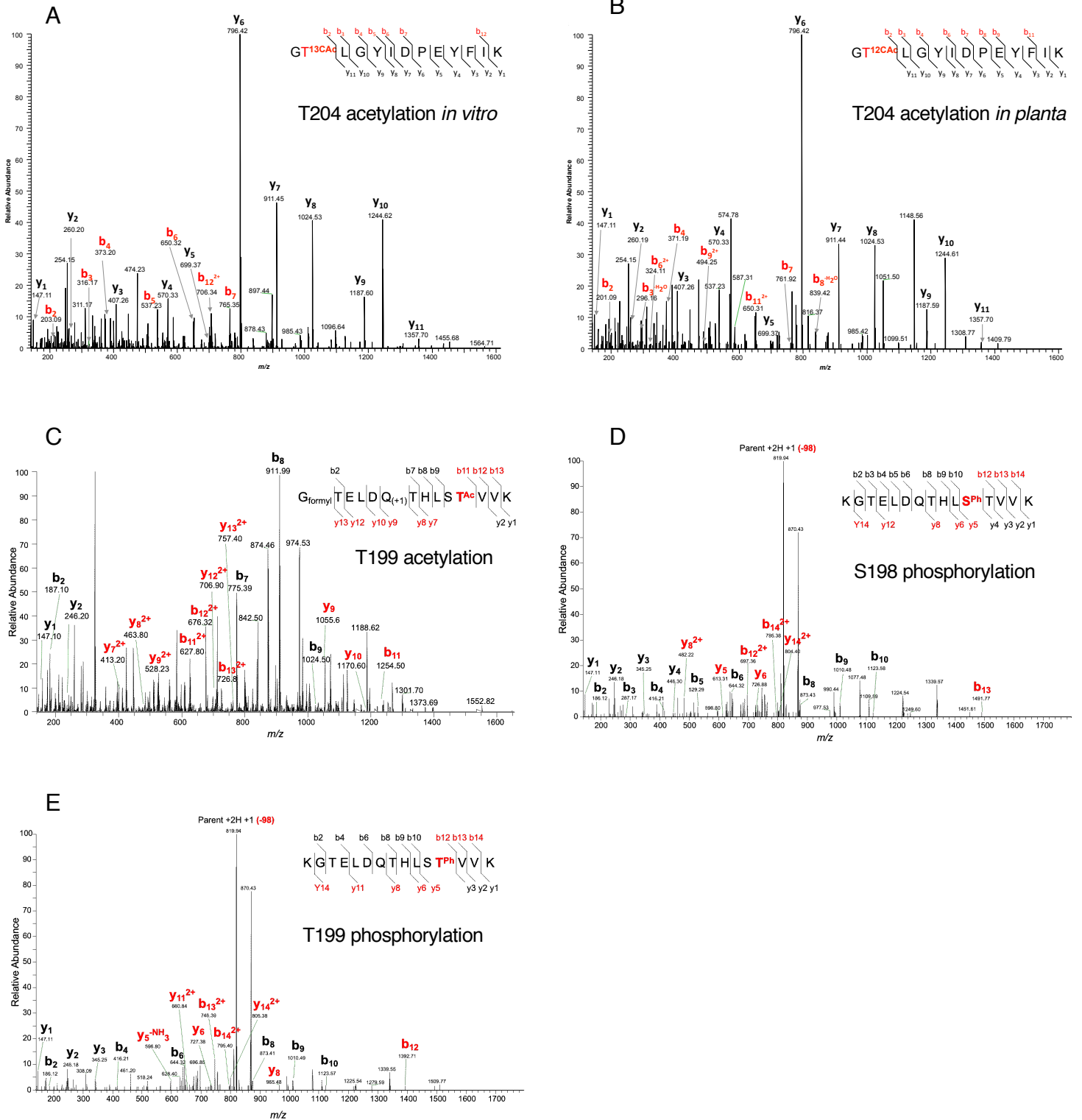
S3 Fig. Pull downs with recombinant tagged proteins to assess interaction between AvrPto1_{PSy} and proteins in the PTO immune pathway. Empty Ni²⁺ resin or immobilized GST were used as negative controls for His- and GST pull downs, respectively. Proteins were detected by Coomassie staining or immunoblotting. (A) Immobilized His-tagged AvrPto1_{PSy} pulled down purified PTO-MBP. (B) Immobilized AvrPto1_{PSy}-His was incubated with FEN-GST, showing no interaction. (C) Immobilized His-tagged AvrPto1_{PSy} was incubated with SIRIN4-1-MBP showing weak interaction in 1 experiment and no interaction in 2 experiments. (D) Immobilized His-tagged AvrPto1_{PSy} was incubated with SIRIN4-2-GST showing weak interaction in 2 of 3 experiments. (E) Immobilized His-tagged AvrPto1_{PSy} was incubated with SIRIN4-3-GST. Interaction was not detected. (F) Immobilized GST-AvrPto1_{PSy} or GST was mixed with His-tagged AvrPtoB_{PSy}, showing that the two effectors interact.

AvrPto1

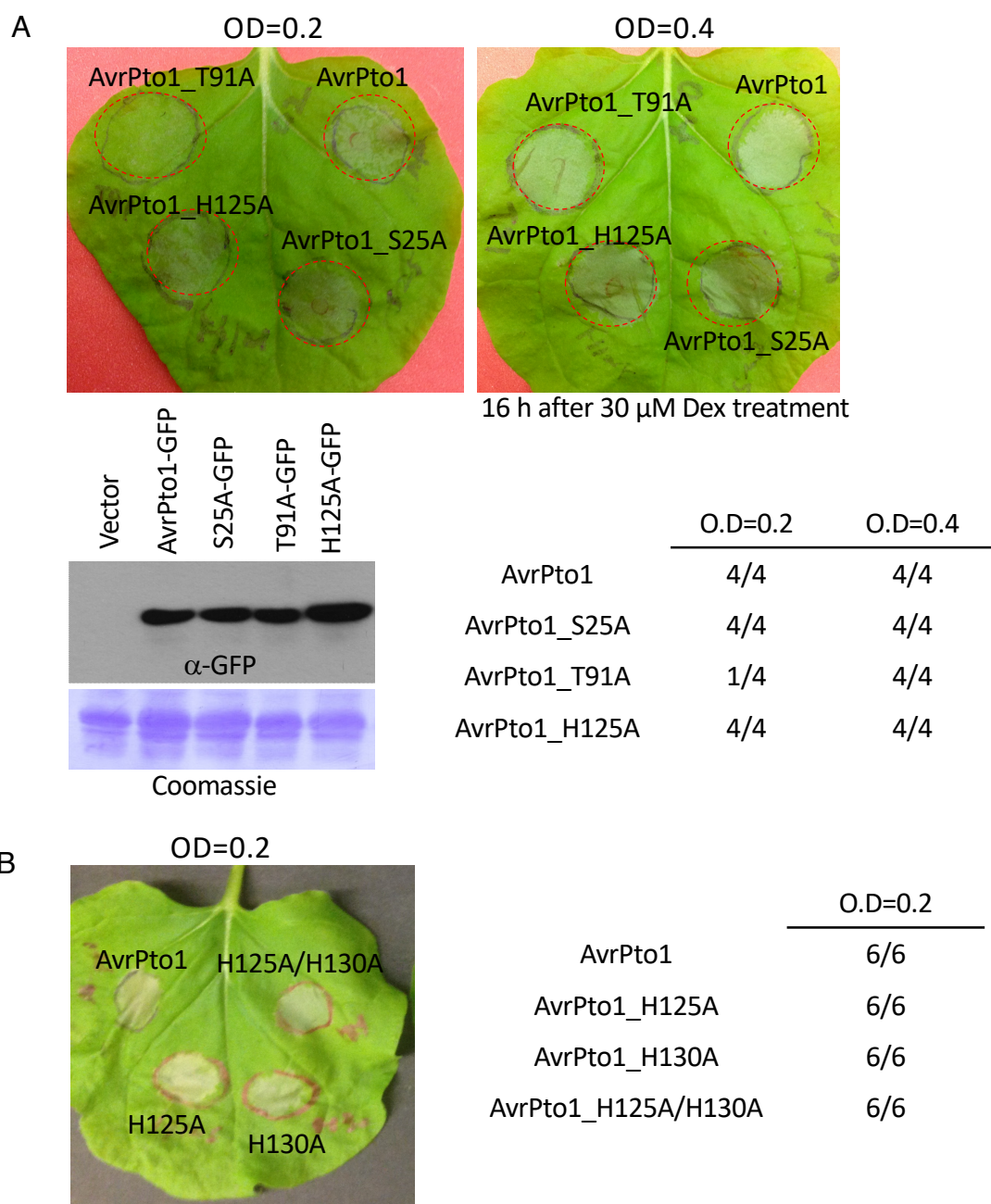


S4 Fig. HopZ3 acetylates multiple sites in AvrPto1_{PSY} important for interaction with PTO and signaling. MS/MS spectra show PTM of AvrPto1_{PSY} expressed in *N. benthamiana* in the presence of HopZ3 or HopZ3_C300A. (A) Evidence of G2 myristoylation. (B) Acetylation of H125 and H130 in the presence of HopZ3. S136 was phosphorylated in both samples (HopZ3 and HopZ3_C300A). (C-E) Acetylation of S147 and S149 in the presence of HopZ3 (C) reduced phosphorylation of these residues. Phosphorylation was observed in the presence of HopZ3_C300A (D-E).

PTO



S5 Fig. HopZ3 acetylates key sites in the activation loop of PTO. PTMs were analyzed using mass spectrometry for PTO after *in vitro* acetylation reaction (A) or co-expressed with HopZ3 or HopZ3_C300A *in N. benthamiana* (B-E). (A-B) In both *in vitro* (A) and *in planta* (B) analyses T204, a key residue in the activation loop of the PTO kinase, was acetylated in the presence of HopZ3. (C) T199 acetylation in the presence of HopZ3. (D-E) Phosphorylation of S198 and T199 in the presence of HopZ3_C300A.



S6 Fig. Effect of mutations of AvrPto1_{PSy} acetylation sites on cell death induction in *N. benthamiana*. AvrPto1_{PSy}-GFP variants were transiently expressed in *N. benthamiana* infiltrated with *Agrobacterium* at OD=0.2 or 0.4 and sprayed with dexamethasone. (A) Only AvrPto1_{PSy}-T91A induced delayed cell death compared to wild-type AvrPto1_{PSy}. All variants were expressed to similar levels. A number of infiltrated areas with cell death per total number of samples is shown in tables. (B) H125, H130 and double mutant of AvrPto1_{PSy} induced cell death in *N. benthamiana*.

```

4_1  MAR-PNVPKFGNWENDNTPTVTVFEKARQTRGT-GKMMNPNDPE-----EN----- 45
4_2  MAR-ANVPKFGNWGNEDNTPTVTVFENARKNRG--GKMINPNDPQ-----EN----- 44
4_3  MAKHSQVPKFGWESEDEDVQYTTYFENAAKGKK--GSKMNPNDPQYLEAKVKGENGTDTV 58
AT   MAR-SNVPKFGNWEAEENVPTAYFDKARKTRAPGSKIMNPNDPE-----YN----- 46
      **: .:*****: * :.: * : : . . :*****: *
4_1  -----PDMFRNLAPPEVAPQSKPKRQ-----TEEPPI 73
4_2  -----PDMF-----PNVAPSSRPKTP-----PTEEPM 66
4_3  RQKPERIASRDDVELRKSTGSPMHPDTMG-----HKVPTYPSQQRHGAKYGGNKSESETM 113
AT   -----SDSQSQAPPHP-----SSRTKP 64
      . . . *:
4_1  GRGGPARQTRDHRLSKEDGEFRQYANSARKESVGRKGANEPHQ-----GRGSNSGRITG 129
4_2  G-METARQTNKRRVSKEDGDFR--ASSPARNEPT-----THQRHGG-----GRGSNSGRPS 114
4_3  KSTEILTPRHERRPSREEGYLRKPTDSLRLNENMGRRTPMESPHHRYGGLSGGATPKRAS 173
AT   EQVDTVRRSREHMRSSREESSELKQFGDAGG-----SSNEAANKR-----QGRAS 107
      .: *::: .: .: .:
4_1  RQSIGSEHSFDKSPLHPHYQAKVSNAGRGVASPAWEGKN-----NSYDSSHGTPG 179
4_2  RQSGSDHSIAKSPLHPNSQAKIS--GRVAASPVWEGKNLYDSSHGTPGRSFESSHATPG 172
4_3  QQSVGPDRSIEHSPLHPHSHGRPGGKGGVSSPSWERKASSE-----GSHGLAPSTPG 226
AT   QNNSYDN---KSPLHKNSYDGTG-----KSRPK 132
      :. : :****: . *
4_1  RSKVK-----QDKSDRGAAPVPRFGEWDENDPQSSADNYTHIFNKFREEKQGNPSGTPS--- 231
4_2  RHQMK-----QESPDRGTVVVPKFGGWDDNDPQDAENYTEVFNKVREQRHVDTGNMPAAGV 227
4_3  RSRLRPVAKGDDTPDDSPAVPKFGDWENDPASAEGYTQIFNKVREEKQTGSAKVPSSST 286
AT   PTNLR---ADESPEKVTVVVPKFGDWENNPSADGYTHIFNKVREERSSSGANVSSSSRT 188
      :.: :.: :.***:* *:*: * :.:***:***:***: :. :
4_1  RTSNNT-QKHNSSEKQRKWCPCPW--- 254
4_2  RTSYST-QRQQRNEKQKSCCFPLW--- 250
4_3  DTSYSNSQKRYGNDSGKGCLCFPWGRS 313
AT   PTHQSS---RNPNNTS-SCCCFGFGGK 211
      * . . : :.: :

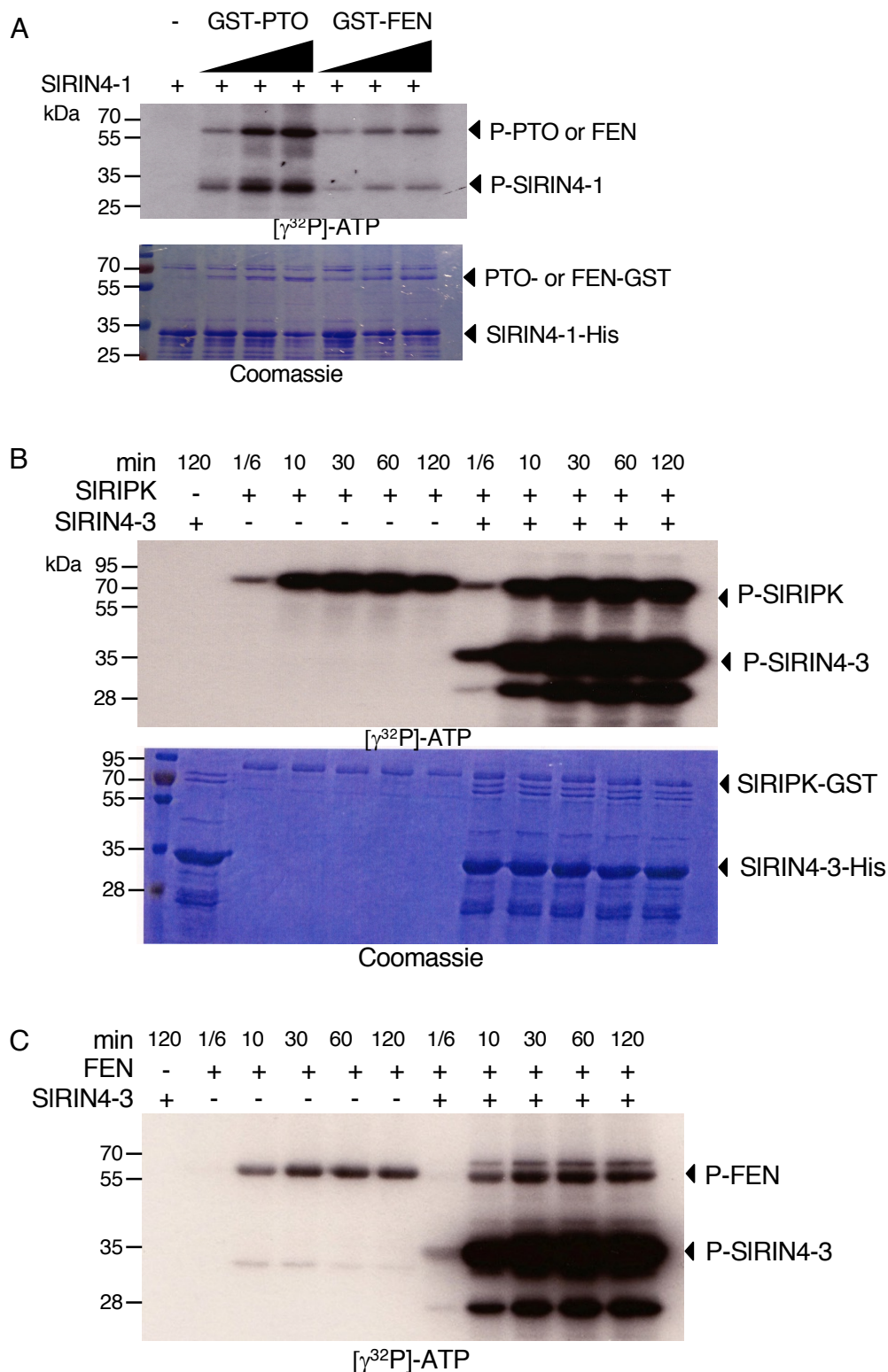
```

S7 Fig. Alignment of tomato RIN4s (4_1, 4_2, 4_3) and Arabidopsis RIN4 (AT).

Modifications in SlRIN4s were determined *in vitro*, modifications in AtRIN4 are from [1] (*in vitro* and *in planta*). Residues acetylated by HopZ3 are bold and highlighted in yellow; phosphorylation sites are underlined; known phosphorylation sites important for signaling (S141, T166) in AtRIN4 [2] are highlighted blue; residues phosphorylated by RIPK in AtRIN4 (T21, S160, T166) [3] are circled in red. * (asterisk) - fully conserved residues, : (colon) - conservation between groups of strongly similar properties, . (period) - conservation between groups of weakly similar properties.

References

1. Lee J, Manning AJ, Wolfgeher D, Jelenska J, Cavanaugh KA, Xu H, et al. Acetylation of an NB-LRR plant immune-effector complex suppresses immunity. *Cell Rep*. 2015;13(8):1670-82. doi: 10.1016/j.celrep.2015.10.029. PubMed PMID: 26586425.
2. Chung EH, El-Kasmi F, He Y, Loehr A, Dangl JL. A plant phosphoswitch platform repeatedly targeted by type III effector proteins regulates the output of both tiers of plant immune receptors. *Cell Host & Microbe*. 2014;16(4):484-94. doi: 10.1016/j.chom.2014.09.004. PubMed PMID: 25299334.
3. Liu J, Elmore JM, Lin ZJ, Coaker G. A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell Host & Microbe*. 2011;9(2):137-46. doi: 10.1016/j.chom.2011.01.010. PubMed PMID: 21320696.



S9 Fig. Kinases in the PTO family can phosphorylate tomato RIN4s. (A) PTO and FEN phosphorylate tomato SIRIN4-1. Kinase assays showing an increasing amount of PTO and FEN autophosphorylation and transphosphorylation of SIRIN4-1. (B-C) SIRIPK and FEN phosphorylate SIRIN4-3. The time course of the *in vitro* kinase reactions is shown. Purified SIRIPK (B) or FEN (C) were incubated in kinase buffer with or without SIRIN4-3 as a substrate. At indicated time points, aliquots of the reaction were taken out and separated by SDS-PAGE.

S1 Table. AvrPto1_{PSy} PTMs *in vitro* and *in planta*.

Site	<i>In vitro</i>		Myr exp 1,2	<i>In planta</i>			
	Ac Z3/CA	Phos		Ac Z3/CA exp 1	exp 2	Phos exp 1	exp 2
G2			+				
S9	2.7	+					
H13	2.2				Z3		
S17	1.5	+			Z3	+	+
S22	5.9					+	+
S25	1.9	+		2	Z3	+	+
T32						+	+
S33						+	+
S46		+		15	5		
S58		+		Z3	Z3		
S59		+		3	14		
S67*	3.8				Z3		
S68*	1.2				0.8	+	+
T79	1.9			Z3	Z3		
H87	0.9			Z3			
T91	2	+		Z3 [#]	18 [#]		
S94	1.6			Z3 [#]	20 [#]		
S111	2.2				Z3		
S114	2.4				Z3		
S117	1.8	+			Z3		
H125	2.2			2 [#]	Z3 [#]		
H130	2.2			2	Z3		
S136						+	+
T142*	Z3	+			Z3	+	
T143*		+				+	
S147	1.6	+		1.6	Z3	+	+
S149	11	+		2	Z3	+	+
T154	19	+					
S158	1.4					+	+
H160	1.4						

PTMs were determined either *in vitro*, using purified recombinant AvrPto1_{PSy} after ¹³C-acetylation by HopZ3/HopZ3_C300A, or *in planta*, by co-expressing AvrPto1_{PSy} and HopZ3/HopZ3_C300A in *N. benthamiana*, followed by immunoprecipitation. Numbers indicate enrichment (fold change) of acetylation in the presence of HopZ3 vs. HopZ3_C300A. **Red shading**: significant (>50%) increase of acetylation with HopZ3. **Blue shading**: significant decrease of phosphorylation *in planta* in the presence of HopZ3. Residues known to be important for AvrPto signaling or interaction with PTO are in **bold**. + indicates phosphorylation found in a recombinant protein (*in vitro*) or *in planta*. Z3: acetylation found only in AvrPto1_{PSy} treated or co-expressed with HopZ3 and not HopZ33_C300A. Ac: acetylation; Phos: phosphorylation; Myr: myristoylation; exp: experiment. *Some spectra do not distinguish these 2 close residues. [#]*In planta* sites with acetylation above 25% in the presence of HopZ3.

S2 Table. PTO PTMs *in vitro* and *in planta*.

Site	<i>In vitro</i>		<i>In planta</i>			
	Ac Z3/CA	Phos	Ac Z3/CA		Phos	
			exp 1	exp 2	exp 1	exp 2
T9*			Z3	1.8		
S11*			Z3	2		
T38		+			+	
K123			Z3	Z3 [#]		
T133		+			+	+
S135					+	+
S137					+	+
T181					+	
T190					+	+
T195		+			+	
S198		+			+	+
T199		+	Z3	Z3 [#]	+	
K202			Z3	1		
T204	Z3		Z3 [#]	Z3 [#]		

PTMs were determined either *in vitro*, using purified recombinant PTO after ¹³C-acetylation by HopZ3/HopZ3_C300A, or *in planta*, by co-expressing PTO and HopZ3/HopZ3_C300A in *N. benthamiana*, followed by immunoprecipitation. Numbers indicate enrichment (fold change) of acetylation in the presence of HopZ3 vs. HopZ3_C300A. **Red shading**: significant (>50%) increase of acetylation with HopZ3. **Blue shading**: significant decrease of phosphorylation *in planta* in the presence of HopZ3. Residues important for PTO signaling or interaction with AvrPto are in **bold**. + indicates phosphorylation found in a recombinant protein (*in vitro*) or *in planta*. Z3: acetylation found only in PTO treated or co-expressed with HopZ3 and not HopZ3_C300A.; Ac: acetylation; Phos: phosphorylation; exp: experiment. *Some spectra do not distinguish these 2 close residues. [#]*In planta* sites with acetylation above 25% in the presence of HopZ3.

S3 Table. SIRIN4s PTMs *in vitro*.

Protein	Site	Ac Z3/CA	Phos
SIRIN4_1	S88	Z3	+
	T128	Z3	
	S153	Z3	
	K167	1.4	
	S188		
	S207	1.4	
	K248	Z3	
SIRIN4_2	T18		+
	K34	2	
	S144		+
	S180		+
	T185		+
	K210	3	
SIRIN4_3	K48	Z3	
	K114	Z3	
	S213		
	T239		
	T276		
	S284		

PTMs were determined using purified recombinant SIRIN4s after *in vitro* ^{13}C -acetylation by HopZ3/HopZ3_C300A. Numbers indicate enrichment (fold change) of ^{13}C -acetylation in the presence of HopZ3 vs. HopZ3_C300A. Z3: acetylation found only in SIRIN4 treated with HopZ3. **Red shading:** significant (>50%) increase of modification with HopZ3. + indicates phosphorylation found in a recombinant protein. Ac: acetylation; Phos: phosphorylation.

S4 Table. SIRIPK PTMs *in vitro*.

Site	Ac Z3/CA	Phos
S10	1.4	+
K17	Z3	
S31		+
H38/H39	Z3	
S44		+
S47		+
S52		+
S56		+
T82	2	+
S83	1.6	+
S86/S87		+
K120	Z3	
H130	2	
T135	2	+
S182	1.4	+
S219	1.5	+
S232		+
T246	1.8	+
H247	2.2	
S249	Z3	+
T250	Z3	+
T255	1.6	+
H256	1.8	
T266		+
S330		+
T354		+
S382		+
T389		+
T396		+
S444		+
T446		+
T452		+

PTMs were determined using purified recombinant SIRIPK after *in vitro* ^{13}C -acetylation by HopZ3/HopZ3_C300A. Numbers indicate enrichment (fold change) of ^{13}C -acetylation in the presence of HopZ3 vs. HopZ3_C300A. Z3: acetylation found only in SIRIPK treated with HopZ3. **Red shading:** significant (>50%) increase of modification with HopZ3. + indicates phosphorylation found in a recombinant protein. Ac: acetylation; Phos: phosphorylation.

S5 Table. Bacterial and yeast strains.

Strain	Description	Antibiotic ^a	Reference/ Source
<i>Escherichia coli</i>			
DH5α	F ⁻ Φ80d <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 λ⁻ gyrA96 relA1</i>	Nal ^R	Invitrogen
BL21 (DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ(DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])		Stratagene
<i>Saccharomyces cerevisiae</i>			
AH109	MAT _a , <i>trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>		Clontech
Y187	MAT _α , <i>ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, MEL1, URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ</i>		Clontech
<i>Pseudomonas syringae</i>			
PsyB728a	<i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	Rif ^R	[1]
PsyB728a Δ <i>hopZ3</i>	HopZ3 knock-out mutant of <i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	Rif ^R	[1]
PsyB728a Δ <i>AvrPto1</i>	AvrB3 knock-out mutant of <i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	Rif ^R	This study
PsyB728a Δ <i>HopZ3</i> Δ <i>AvrPto1</i>	HopZ3/AvrPto1 double knock-out mutant of <i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a	Rif ^R	This study
<i>Agrobacterium tumefaciens</i>			
GV3101 (pCH32)	Used for <i>Agrobacterium</i> -mediated transient expression in <i>N. benthamiana</i>	Gm ^R /Rif ^R	
C58C1 (pMP90)	Used for <i>Agrobacterium</i> -mediated transient expression in <i>N. benthamiana</i>	Tet ^R	

^a Nal^R, nalidixic acid resistance; Rif^R, rifampicin resistance; Gm^R, gentamycin resistance; Tet^R, tetracycline resistance.

References

1. Vinatzer BA, Teitzel GM, Lee MW, Jelenska J, Hotton S, Fairfax K, et al. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol Microbiol.* 2006;62(1):26-44. doi: 10.1111/j.1365-2958.2006.05350.x. PubMed PMID: 16942603.

S6 Table. Plasmid vectors.

Plasmid	Parent vector	Description	Antibiotic ^a	Reference
pBAV154	pTA7001	Gateway-compatible plant expression binary vector (<i>dex</i> inducible promoter and C-terminal HA-tag)	Km ^R / Cm ^R /BASTA ^R	[1]
pBAV150	pTA7001	Gateway-compatible plant expression binary vector (<i>dex</i> inducible promoter and C-terminal GFP-tag)	Km ^R / Cm ^R /BASTA ^R	[1]
pTA7001		Vector control for <i>Agrobacterium</i>		[1]
pBAV226	pME6010	Gateway-compatible <i>P. syringae</i> expression vector (<i>nptII</i> promoter, low copy plasmid, C-terminal HA-Tag)	Tet ^R / Cm ^R	[1]
pME6010		Vector control for <i>P. syringae</i>		[1]
pMTN1907		Gateway™ vector for making unmarked deletions in <i>P. syringae</i> . SacB cassette	Tet ^R	[2]
pDONR207		Gateway™ cloning vector	Gm ^R / Cm ^R	Invitrogen
pET DUET		T7 promoter, N-terminal His-tag or C-terminal S-tag	Amp ^R	Novagen
pET28a		T7 promoter, N-terminal His-tag and T7-tag, optional C-terminal His-tag	Km ^R	Novagen
pGEX 4T-1		tac promoter and N-terminal GST-tag with thrombin cleavage site	Amp ^R	Amersham/ Pharmacia
pGEX 4T-3 with rTEV	pGEX 4T-3	tac promoter and N-terminal GST-tag, pGEX 4T-3 with rTEV site	Amp ^R	from K. Orth (UT Southwestern)
pMAL-c5X		tac promoter and N-terminal MBP fusion	Amp ^R	NE BioLabs
pG005	pGreen	BIFC gateway binary vector 35S promoter, GW, YFP N-terminus	Sp ^R / Cm ^R /Hyg ^R	NASC
pG006	pGreen	BIFC gateway binary vector, 35S promoter, GW, YFP C-terminus	Sp ^R / Cm ^R /Hyg ^R	NASC
pLaw10	pGBK T7	Yeast two-hybrid GAL4 BD vector, ADH1 promoter, N-terminal c-Myc tag. Gateway compatible derivative of pGBKT7 (Clontech).	Km ^R / Cm ^R TRP	[3]
pLaw11	pGAD7	Yeast two-hybrid GAL4 AD vector, ADH1 promoter, N-terminal HA tag. Gateway compatible derivative of pGADT7 (Clontech).	Amp ^R / Cm ^R /LEU	[3]

^a Amp^R, ampicillin resistance; BASTA^R, BASTA (glufosinate ammonium) resistance; Cm^R, chloroamphenicol resistance; Gm^R, gentamycin resistance; Hyg^R, Hygromycin resistance; Km^R, kanamycin resistance; Tet^R, tetracycline resistance; Sp^R, spectinomycin resistance.

References

1. Vinatzer BA, Teitzel GM, Lee MW, Jelenska J, Hotton S, Fairfax K, et al. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol Microbiol.* 2006;62(1):26-44. doi: 10.1111/j.1365-2958.2006.05350.x. PubMed PMID: 16942603.
2. Baltrus DA, Nishimura MT, Dougherty KM, Biswas S, Mukhtar MS, Vicente J, et al. The molecular basis of host specialization in bean pathovars of *Pseudomonas syringae*. *Mol Plant Microbe Interact.* 2012;25(7):877-88. doi: 10.1094/MPMI-08-11-0218. PubMed PMID: 22414441.
3. Lee J, Manning AJ, Wolfgeher D, Jelenska J, Cavanaugh KA, Xu H, et al. Acetylation of an NB-LRR plant immune-effector complex suppresses immunity. *Cell Rep.* 2015;13(8):1670-82. doi: 10.1016/j.celrep.2015.10.029. PubMed PMID: 26586425.

S7 Table. PCR primer sequences used in this study.

Primer name	Primer sequence (5'→3')
AvrPto1 _{Psy} 5'F1	AAAAAGCAGGCTTCAACTGGTTGCGCAAGACTGA
AvrPto1 _{Psy} 5'R1	TACGGTGCACATATATTTCCCATTCGTATACCC
AvrPto1 _{Psy} 3'F2	AATATATGTGCGACCGTAACTGGTAATGA
AvrPto1 _{Psy} 3'R2	AGAAAGCTGGGTCTACAGCGACATGCTGGAAC
PTO-EcoRI-F	GGGAATCATGGGAAGCAAGTATTCTAA
PTO-Sall-R	AGCTTGTCGACAATAACAGACTCTTGGAGAC
PTO T204R-F	GTGAAAGGA <u>A</u> ACTCGGCTAC
PTO T204R-R	GTAGCCGAGTCTTCCTTTTAC
PTO T204N-F	GTGAAAGGA <u>A</u> ATCTCGGCTAC
PTO T204N-R	GTAGCCGAGATTTCTTTTAC
FEN-EcoRI-F	GGGAATTCATGGGAAGCAAGTATTCC
FEN-Sall-R	AGCTTGTCGACGTTTCAGGATCATCTTGAATGG
FEN N202T-F	ACAGTAGTGAGAGGA <u>A</u> CTATAGGCTACATTGCC
FEN N202T-R	GGCAATGTAGCCTATAGTTCTCTCACTACTGT
AvrPto1 _{Psy} -EcoRI-F	CCGAATTCGATGGGAATATATGTGTC
AvrPto1 _{Psy} -Sall-R	CTTGTCGACCCAGTTACGGTGC GGCT
AvrPto1 _{Psy} T91A-F	CAGCATAGGTTTCATGG <u>C</u> GGAGCGTCAGGA
AvrPto1 _{Psy} T91A-R	TCCTGACGCTCCCGCCATGAACCTATGCTG
AvrPto1 _{Psy} H125A/H130A-F	GAAGCTCTGCAGG <u>C</u> CGCAATGGGTATCG <u>C</u> CGCTGACATCCCA
AvrPto1 _{Psy} H125A/H130A-R	TGGGATGTCAGCGGCGATACCCATTGCGGCTGCAGAGCTTC
SIRIPK-Sall-F	CGGGTCGACATGGCTACTTGCGGAATTGACT
SIRIPK-Not1-R	GGGCGGCCGCTATAAGAATTTAGTTTGTTTG
SIRIN4-1-BamHI-F	GTGGATCCGATGGCTCGTCCAAATGTCCC
SIRIN4-1-XhoI-R	CCGGCTCGAGTTACCAGGGACAACAACACC
SIRIN4-2-BamHI-F	GTGGATCCGATGGCTCGTGCAAATGTG
SIRIN4-2-XhoI-R	CCGCTCGAGTCACCACAAAGGAAAGCA
SIRIN4-3-BamHI-F	GTGGATCCGATGGCAAAACACTCACAA
SIRIN4-3-XhoI-R	CCGCTCGAGTCAATCCGACCCCATGGGAAACAT
AvrPto1 _{Psy} -DONR-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGAAATATATGTGTCGGC
AvrPto1 _{Psy} -DONR-R	GGGACCACTTTGTACAAGAAAGCTGGGTCCCAGTTACGGTGCGGGCTAGG
PTO-DONR-F	AAAAAGCAGGCTTCATGGGAAGCAAGTATTCTAA
PTO-DONR-R	AGAAAGCTGGGTCAATAACAGACTCTTGGAGAC
FEN-DONR-F	AAAAAGCAGGCTTCATGGGAAGCAAGTATTCC
FEN-DONR-R	AGAAAGCTGGGTCTTCAGGATCATCTTGAATGG
SIRIPK-DONR-F	AAAAAGCAGGCTTCATGGCTACTTGCGGAATTGACT
SIRIPK-DONR-R	AGAAAGCTGGGTCATAAGAATTTAGTTTGTTTG
SIRIN4-1-DONR-F	AAAAAGCAGGCTATGGCTCGTCCAAATGTCCC
SIRIN4-1-DONR-R	AGAAAGCTGGGTCCCAGGGACAACAACACCA
SIRIN4-2-DONR-F	AAAAAGCAGGCTATGGCTCGTGCAAATGTG
SIRIN4-2-DONR-R	AGAAAGCTGGGTCCCACAAAGGAAAGCAGCA
SIRIN4-3-DONR-F	AAAAAGCAGGCTATGGCAAAACACTCACAA
SIRIN4-3-DONR-R	AGAAAGCTGGGTCACTCCGACCCCATGGGAA

Mutated codons are underlined.