Plants are resistant to most microbial species due to nonhost resistance (NHR), providing broad-spectrum and durable immunity. However, the molecular components contributing to NHR are poorly characterised. We address the question of whether failure of pathogen effectors to manipulate nonhost plants plays a critical role in NHR. RxLR (Arg-any amino acid-Leu-Arg) effectors from two oomycete pathogens, Phytophthora infestans and Hyaloperonospora arabidopsidis, enhanced pathogen infection when expressed in host plants (Nicotiana benthamiana and Arabidopsis, respectively) but the same effectors performed poorly in distantly related nonhost pathosystems. Putative target proteins in the host plant potato were identified for 64 P. infestans RxLR effectors using yeast 2-hybrid (Y2H) screens. Candidate orthologues of these target proteins in the distantly related non-host plant Arabidopsis were identified and screened using matrix Y2H for interaction with RxLR effectors from both P. infestans and H. arabidopsidis. Few P. infestans effector-target protein interactions were conserved from potato to candidate Arabidopsis orthologs (cAtOrths). However, there was an enrichment of H. arabidopsidis RxLR effectors interacting with cAtOrths. We expressed the cAtOrth AtPUB33, which unlike its potato orthologue did not interact with P. infestans effector PiSFI3, in potato and Nicotiana benthamiana. Expression of AtPUB33 significantly reduced P. infestans colonization in both host plants. Our results provide evidence that failure of pathogen effectors to interact with and/or correctly manipulate target proteins in distantly related non-host plants contributes to NHR. Moreover, exploiting this breakdown in effector–non-host target interaction, transferring effector target orthologues from non-host to host plants is a strategy to reduce disease.

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Significance

Plant nonhost resistance (NHR) prevents infection by all members of most microbial species, but its molecular mechanisms are not well understood. We found that effector proteins from the potato blight pathogen Phytophthora infestans, which enhance infection in host plants, fail to enhance susceptibility in nonhost Arabidopsis. These P. infestans effectors often failed to interact with Arabidopsis orthologs of their potato target proteins, whereas many interactions were detected between these Arabidopsis orthologs and effectors from its adapted pathogen Hyaloperonospora arabidopsidis. Thus, breakdown in effector–target interactions in distantly related non-host plants is likely a key component of NHR. Importantly, we demonstrate that exploiting this breakdown and expressing nonhost target orthologs in host plants provide a strategy to prevent crop disease.
techniques (11). Indeed, it is possible to carry out high-throughput yeast two-hybrid (Y2H) screens, whereby a large complement of proteins of interest can be screened simultaneously against an array of potential targets (12). In this way, candidate cytoplasmic effector sets from adapted Arabidopsis pathogens (the oomycete Hyaloperonospora arabidopsidis, the bacterium Pseudomonas syringar pathovar tomato, and the fungus Golovinomyces orontii [Gol]) were screened against >8,000 Arabidopsis proteins, identifying candidate host protein targets potentially involved in the defense response to these pathogens (13, 14). Interestingly, these screens revealed that some effectors from these three pathogens, representing different evolutionary kingdoms of life, apparently converged on common host protein targets, suggesting that there may be shared strategies to suppress or modify host processes (13, 14). In contrast to such large-scale screens, there is also a body of work with more in-depth analysis of how effectors from different pathogens suppress and/or manipulate host defenses (11, 15). Bacterial type III effectors often possess enzyme activity and target components of plant immunity, such as pattern recognition receptors (PRRs); coreceptors, like BAK1; and signaling components, such as BIK1 and mitogen-activated protein kinases (MAPKs) (16). Many of the fungal effectors whose mode of action is known are apoplastic proteins that inhibit plant proteases or have chitin binding activity (17). However, many fungal and oomycete cytoplasmic effectors target diverse pathways and processes from signal transduction to RNA processing and silencing, hormone signaling, and secretion, often altering posttranslational modifications, such as ubiquitination and phosphorylation (11). Some oomycete effectors have evolved to use the activity of their host targets for infection; such targets can be regarded as susceptibility (S) factors (11, 18).

In light of these developments, candidate cytoplasmic effectors from the potato late blight pathogen Phytophthora infestans and Arabidopsis pathogen H. arabidopsidis were studied to determine whether they displayed activity as virulence factors in both host and distantly related nonhost pathosystems. P. infestans and H. arabidopsidis are both oomycetes that express the cytoplasmic RxLR (Arg–any amino acid–Leu–Arg) motif containing effectors during host colonization, several of which have been functionally characterized (19–22). P. infestans infects potato, tomato, eggplant, and the model solanaceous plant Nicotiana benthamiana (23) but not the model plant Arabidopsis, a member of the Brassicaceae (24). Arabidopsis can be artificially made to allow P. infestans to complete its infection cycle in laboratory conditions but only where plant defenses have been suppressed by preinfestation with Allbugo laibachii (25, 26). Attempts to render Arabidopsis susceptible to P. infestans by genetic means have not been successful. In contrast, H. arabidopsidis infects Arabidopsis but not members of the Solanaceae (27). Understanding the mechanisms by which Arabidopsis and solanaceous plants are resistant to P. infestans and H. arabidopsidis, respectively, will unlock potential to exploit NHR for crop protection.

In this study, we expressed RxLR effectors from H. arabidopsidis and P. infestans in their nonhost plants N. benthamiana and Arabidopsis, respectively, to determine whether these effectors contribute to pathogen virulence in these plants. Y2H assays were employed to identify proteins (potential targets) in potato that interacted with a selection of 64 P. infestans (Pi) RxLR effectors. We then tested whether the effectors from P. infestans were able to retain interaction with candidate Arabidopsis thaliana orthologs (cAtOrths) of their potato host targets as well as identifying H. arabidopsidis RxLR effectors (HaRxLs) that were able to interact with these Arabidopsis proteins. Finally, we tested whether expression in wild-type Solanaceae plants of P. infestans “target orthologs” from the nonhost Arabidopsis, which evade interaction with PiRxLR effectors, reduced the capacity of P. infestans to colonize its hosts.

**Results**

RxLR Effectors Are Unable to Enhance Pathogen Colonization in Distantly Related Nonhost Plants. To investigate whether effectors contribute to pathogenicity in both host and nonhost pathosystems, we selected RxLR effectors from two oomycete pathogens, P. infestans and H. arabidopsidis; expressed them individually in their host pathosystems (PiRxLRs in N. benthamiana; HaRxLs in Arabidopsis) and in their respective nonhost pathosystem (PiRxLRs in Arabidopsis; HaRxLs in N. benthamiana); and assessed whether expression enhanced colonization by host-adapted pathogens (P. infestans on N. benthamiana and H. arabidopsidis on Arabidopsis). Fifteen PiRxLR effectors were selected on the basis that they had been shown previously to enhance P. infestans colonization (28). They were expressed transiently (with green fluorescent protein [GFP] tag) in N. benthamiana, and plants were challenged 1 d later with P. infestans zoospores. Fig. 1A shows that as shown previously (28), when each of the PiRxLR effectors is expressed lacking their signal peptide, with N-terminal GFP tag, there is a significant increase in P. infestans lesion sizes, between 1.2- to 3.5-fold higher than controls expressing GFP alone as measured by lesion diameter. Each GFP–effector fusion protein was stable in planta (SI Appendix, Fig. S1A). We then expressed 10 HaRxLs in N. benthamiana. In contrast to the PiRxLRs, only three HaRxLs significantly enhanced P. infestans colonization of N. benthamiana compared with the GFP control, and those that reproducibly enhanced colonization were only modestly able to do so (between 1.2- and 1.5-fold) when compared with the majority of P. infestans effectors (Fig. 1B). All of those that failed to enhance P. infestans lesion sizes were nevertheless shown to be expressed as intact haemagglutinin (HA)-fusion proteins (SI Appendix, Fig. S1B). We did not observe any hypersensitive response (HR) in N. benthamiana in response to the HaRxL effector expression, so they are unlikely to be recognized by corresponding R proteins. The HaRxL effectors were selected on the basis of their ability to enhance Arabidopsis susceptibility to H. arabidopsidis when expressed in planta (refs. 19 and 29 and this study) rather than on similarity to other oomycete effectors at the sequence level. The few RxLR effectors that are conserved between H. arabidopsidis and Phytophthora species have been shown to suppress immunity in a range of host and nonhost plants (30, 31).

To perform reciprocal experiments, transgenic Arabidopsis plants individually expressing the 10 HaRxLs (SI Appendix, Fig. S2A) (lines are described in ref. 19) were challenged by H. arabidopsidis. At least two transgenic lines expressing each H. arabidopsidis effector were selected and shown to be significantly more susceptible to H. arabidopsidis infection as measured by sporangiophore counts, compared with control plants (Fig. 1C). In contrast, when transgenic Arabidopsis were generated expressing the 15 RxLR effectors from the nonhost pathogen P. infestans (SI Appendix, Fig. S2B), only one line expressing effector Pi09218 showed a significant enhancement in colonization by H. arabidopsidis compared with control plants, but this was not shared by the other two lines expressing this effector (Fig. 1D). By contrast, lines expressing two of the effectors (Pi04089 and Pi10654) showed significantly reduced H. arabidopsidis colonization, suggesting that activity of these effectors was detrimental to H. arabidopsidis infection. In order to understand this, we investigated whether these lines had an altered developmental phenotype. Lines expressing Pi04089 were found to display an early...
flowering phenotype (SI Appendix, Fig. S3), suggesting that overexpression of the *P. infestans* effector modifies this developmental process. Taken together, these data suggest that effectors perform better to enhance pathogen virulence in host plants rather than in a distantly related nonhost plant. This could be due to differences between each pathogen in their requirements for host manipulation to create a susceptible environment. However, it could also be because the effector has made an untargeted (or off-target) change to a host protein. Moreover, it could also be explained by failure of the effectors to either interact with or to appropriately manipulate the activities of target proteins in the nonhost plants. To explore the latter, we sought to identify interacting host potato proteins of *P. infestans* RxLR effectors.

**PiRxLR Effectors Interact with a Range of Host Proteins.** To identify candidate host targets of *P. infestans* effectors in potato, 64 PiRxLR effectors (Dataset S1), including the 15 in Fig. 1, were screened individually against a potato complementary DNA (cDNA) Y2H library (Dataset S2), here referred to as cY2H. This cY2H library was made from cDNA prepared from both compatible and incompatible potato-*P. infestans* interactions (32), and it has been extensively used to identify targets of PiRxLR effectors that have been verified in planta (32–44). The 64 PiRxLR effectors were prioritized based on being up-regulated during infection in a range of *P. infestans* genotypes, having diverse subcellular localizations, and possessing the capacity to enhance *P. infestans* colonization of *N. benthamiana* (28). Of the effectors screened, 24 (38%) did not reveal any interacting potato protein following the cY2H screens, despite a high number of yeast transformants (>1 × 10^6) being obtained in each case. The remaining 40 effectors revealed a total of 169 interacting potato proteins (representing 215 interactions) (Fig. 2A, Dataset S2A, and SI Appendix, Fig. S4). Many effectors interacted with more than one potato protein (Fig. 2B), while a subset of potato proteins interacted with multiple effectors (Fig. 2C and Dataset S2B).

A full list of the RxLR effectors screened and the targets identified is shown in Dataset S2. Twenty-seven effectors (42%) shared a subset of their interacting host protein candidate targets with other effectors (Fig. 2, Dataset S2B, and SI Appendix, Fig. S4). The sequence similarity between PiRxLR effectors has been investigated previously using Markov clustering (MCL) (8), prompting us to see whether PiRxLRs with common host interactors were related at the primary sequence level. Little evidence was found for this; only 6 of the 27 effectors that shared host protein targets were from the same PiRxLR families (Pi17309 and Pi17316 in RxLRfam1, Pi16663 and Pi22922 in RxLRfam2, Pi21388 and PiIPIO4 in RxLRfam54) (Dataset S2B), suggesting that, in general, sequence-unrelated effectors may interact with shared target proteins by means of convergent evolution. In other pathosystems, convergence of unrelated effectors from one pathogen onto common host proteins was previously described (14). Using random sampling from an estimated 10,000-protein search space in the cDNA library, we demonstrate that the convergence of different PiRxLRs effectors on common host proteins is highly significant (*P < 0.0001, empirical test*) (SI Appendix, Fig. S5). Importantly, it was also shown previously that the extent of convergence correlates with the ability to observe immune phenotypes in *Arabidopsis* genetic knockout lines (14), suggesting that the redundantly targeted potato proteins are likely important for infection. In addition to this convergence, we also noted that effectors from the same family tended to interact with different host proteins, potentially highlighting divergent evolution to acquire new targets (Dataset S2C). For example, Pi07387 and Pi22926, which both belong
to RxLRfam52, interact with 18 distinct proteins and show no interactors in common with each other.

**PiRxLR Effectors Are Often Unable to Maintain Target Interactions in the Nonhost Arabidopsis.** To determine whether *P. infestans* effectors maintain interactions with candidate orthologs of their targets in a distantly related nonhost plant, reciprocal best blast hit (RBBH) or best blast hit (BBH) analysis, alongside phylogenetic analyses of orthology available in EnsemblPlants (plants.ensembl.org/index.html), was employed to identify AtOrths of the putative potato effector targets (Dataset S3). Of 159 cAtOrths, 100 were successfully cloned full-length (FL) de novo, and a further 16 were found to be present in the existing Y2H *Arabidopsis* open reading frame collection (ORFeome) (13, 14), resulting in a matrix candidate AtOrth library (ortholog matrix orthologue yeast two-hybrid [MoY2H]) of 116 *Arabidopsis* proteins. This resulted in a “testable” network of 153 PiRxLR–cAtOrth interactions based on interactions between 40 unique PiRxLRs and 116 unique target candidates. The 116 cAtOrths were screened against the original 64 *P. infestans* effectors (Fig. 3A, Dataset S3, and SI Appendix, Fig. S6A). In addition, they were screened for interaction with 169 HaRxL effectors (Dataset S1B). Thirty-four of the HaRxL effectors were represented by more than one allele, cloned from distinct *H. arabidopsidis* genotypes (Dataset S1B).

The 116 cAtOrths were cloned into the same Y2H system used in previous matrix Y2H screens (13, 14) to add to those existing screening resources and to facilitate comparisons with those studies. Of the 116 potato cDNAs identified in the cY2H screen (Dataset S2), 42% encoded FL proteins, and a further 18% were missing less than the first 20 amino acids (Dataset S3). Previously, where partial sequences were recovered in the potato cY2H screen, FL sequences that were subsequently cloned retained interactions with corresponding effectors using the Y2H assay (34, 35, 37, 39, 43, 44). To further test this, we selected effector Pi06099, which interacted with a partial StPhyB sequence in the potato cY2H library screen and also interacted with FL AtPhyB in the MoY2H system (Dataset S3). We observed that FL StPhyB and AtPhyB sequences each interacted specifically with Pi06099 in the cY2H system, demonstrating that we can reproduce results in each Y2H system (SI Appendix, Fig. S7A). We also verified that Pi06099 interacts with both FL StPhyB and AtPhyB in planta using coimmunoprecipitation (coIP) (SI Appendix, Fig. S7B).

Eighty-nine of the 116 cAtOrths tested did not interact with PiRxLRs; thus, we observed that the majority of *P. infestans* effector–target interactions in potato were apparently not retained in the nonhost *Arabidopsis*, as analyzed using Y2H (126 of 153 interaction pairs that were tested) (Fig. 3B). We selected the interaction between Pi21388 (ipi01/AvrBlb1) and CML36, confirming that the effector interacts with FL StCML36 but not with AtCML36 in both the cY2H (SI Appendix, Fig. S8A) and MoY2H (SI Appendix, Fig. S8B) systems. Moreover, we confirmed that Pi21388 interacts with StCML36 in planta, but not with AtCML36, using coIP (SI Appendix, Fig. S8C).

The inclusion of 169 *H. arabidopsidis* effectors in the MoY2H screen resulted in a complex network of interactions (Fig. 3B, Dataset S3, and SI Appendix, Fig. S6B), which could be separated into four broad categories (Fig. 3C) based on the interaction status of the AtOrth. In total, 27 of the AtOrths interacted with at least one PiRxLR effector (the original one that interacted with the potato ortholog and/or a different PiRxLR). Of these, 13 AtOrths did not interact with an HaRxL (category 1), while 14 of the AtOrths interacted with effectors from both pathogens (category 2). In contrast, 15 AtOrths did not interact with a PiRxLR...
but did interact with at least one HaRxL (category 3). There were also 74 AtOrths where no interactions (category 4) were detected, suggesting that these Arabidopsis proteins are likely to be significantly sequence divergent to evade the P. infestans effector and that, moreover, the Arabidopsis protein is perhaps not important for host defense to H. arabidopsidis as no equivalent interaction is seen with the HaRxLs tested (Fig. 3C).

Strikingly, combining categories 2 and 3 reveals a total of 29 AtOrths that interact with H. arabidopsidis effectors (Fig. 3C), some of which interact with multiple HaRxLs (Fig. 3D). In total, 52 distinct HaRxLs interacted with AtOrth candidate targets (Dataset S3). In some cases where HaRxLs were represented by more than one allele, the alleles interacted with a specific AtOrth. Examples include HaRxL15 alleles that interact with the AtNAC17 transcription factor and the previously reported interactions between HaRxL106 and importin α-isomers (45). Moreover, six independent ATR1 alleles interact with a mitochondrial small heat-shock protein, perhaps emphasizing this as a strong candidate target for future study (Dataset S3), especially given that it was also an interactor of effectors from Go (14). Many of these are candidate targets of H. arabidopsidis effectors, which have no known role in defense against H. arabidopsidis. Furthermore, the overlap of PiRxLR–potato/AtOrth and HaRxL–AtOrth interactions suggests that perhaps P. infestans and H. arabidopsidis as oomycete pathogens share common strategies to manipulate plants. We investigated whether the number of interactions between H. arabidopsidis effectors and AtOrths was higher than expected by chance, indicating that by selecting orthologs of effectors targets from a nonrelated pathogen, we are enriching for host–effector targets/defense components in Arabidopsis. Compared with previous unbiased matrix ORFeome Y2H screens to find plant pathogen effector–target interactions (13, 14), the data from our targeted screen show a significant increase in HaRxL interactions (Fig. 3E). This suggests that the P. infestans–potato cY2H interactome is significantly enriched in potential potato orthologs of Arabidopsis proteins that are targets of HaRxL effectors. We performed a gene ontology (GO) term analysis for biological processes of the Arabidopsis AtOrths (Dataset S3). Twelve GO terms were enriched among the AtOrths (SI Appendix, Fig. S9). Interestingly, these include “Golgi to plasma membrane transport” and “vesicle-mediated transport to plasma membrane,” in agreement with a recent report that P. infestans effector targets may be enriched for vesicle trafficking (46). These data indicate that the common host–pathogen interaction interface described in Weßling et al. (14) as being converged on by evolutionarily diverse pathogens may exist in similar form in diverse plant host species.

Expression of AtOrths in a Susceptible P. infestans Host Plant Can Alter Resistance. The P. infestans and H. arabidopsidis effectors tested performed poorly in the nonhost system (Fig. 1), and in the majority of cases, the P. infestans effectors were unable to maintain their interactions with cAtOrth (Fig. 3B and Dataset S3). Hence, we hypothesized that if the AtOrth did not interact with or could not be correctly manipulated by the corresponding P. infestans RxLR effector, the AtOrth could compensate for the loss of the native host protein being targeted by the effector, provided it retained its function. To test this, a selection of AtOrths was transiently expressed in N. benthamiana to assess whether they could alter susceptibility to P. infestans in a host plant. Protein expression for each AtOrth is shown in SI Appendix, Fig. S10. The AtOrths were selected to represent a range of interaction categories (Table 1). Of the 23 AtOrths selected for expression in N. benthamiana, 17 represented categories 3 and 4 (i.e., no interaction of the P. infestans effector with the Arabidopsis protein). Only expression of AT5G15270 and AT2G45910, both category 4 interactors, significantly altered P. infestans infection levels (Fig. 4). AT5G15270 overexpression enhanced P. infestans colonization compared with the control. Interestingly the potato equivalent, StKRP1, acts as an S factor, and its overexpression also boosts P. infestans colonization of N. benthamiana (33). Although

Fig. 3. Using a library based on interacting non-host orthologs enriches for pathogen effector–protein–protein interactions (PPIs). (A) Network diagram representation of the candidate AtOrths (green circles) of potato cY2H targets, which were identified and cloned. Yellow circles show the PiRxLRs anticipated to interact with cAtOrths based on interactions with potato counterparts (Fig. 2). Straight edges indicate PPIS, and gray circles and edges indicate noncloned orthologs and untestable interactions, respectively. (B) Network diagram representing the newly identified MoY2H PPIS detected between cAtOrths (green circles) and HaRxLs (blue circles) or PiRxLRs (yellow circles). AtOrths not cloned or not interacting (gray circles) and untestable or no interactions detected (gray edges) are also shown. (C) Schematic representation of the four different categories of PPIS identified alongside an explanation and the numbers involved. Colors and shapes are as described in B. Arabidopsis thaliana (At), Hyaloperonospora arabidopsidis (Ha), Solanum tuberosum (St). (D) Histogram showing the count of AtOrths that interact with a given number of HaRxL effectors. The graph shows a significant (P < 1e-05) increase in HaRxL interactions observed (red arrow) compared with the level expected by random sampling modeling analysis.
Atg5i15270 does not interact with Pi04089 in either the cY2H (SI Appendix, Fig. S11A) or MoY2H (SI Appendix, Fig. S11B) systems and did not interact in planta using coIP (SI Appendix, Fig. S11C). StKRBP1 is able to homodimerize and also, to weakly coimmunoprecipitate Atg5i15270 (SI Appendix, Fig. S11D). Moreover, StKRBP1 and Atg5i15270 colocalize at nuclear speckles (SI Appendix, Fig. S11E). This indicates that the candidate orthologs may form part of the same complex in planta. In contrast, transient overexpression of AT2G45910 (AtPUB33) resulted in a significant decrease in P. infestans colonization (Fig. 4), and this gene was thus studied in greater detail.

Expression of AtPUB33 Reduces P. infestans Colonization of Host Plants. AtPUB33 is an E3 ubiquitin ligase but is unique within the class of plant U-box (PUB) domain–containing proteins as it also contains a kinase domain. This study and previous work have shown that the protein ortholog of AtPUB33, StUUBK (PGSC0003DMT400000146), is targeted by the P. infestans effector Pi06087 (PiSFI3/PexRD16) (34). As AtPUB33 belonged to category 4 (no interactors from either pathogen) in the MoY2H screen, Y2H pairwise tests were carried out confirming that AtPUB33 does not interact with Pi06087, whereas StUUBK does (SI Appendix, Fig. S12). To verify the data from the transient assay, stable transgenic lines overexpressing AtPUB33 were constructed in both P. infestans host species: cMYC-AtPUB33 in N. benthamiana and untagged AtPUB33 expression in potato (SI Appendix, Fig. S13). Five independent transgenic lines were selected for each plant species where there was production of detectable myc-PUB33 protein (SI Appendix, Fig. S13 A and B) or detectable AtPUB33 transcript (SI Appendix, Fig. S13C). The transgenic potato and N. benthamiana plants were subsequently challenged with P. infestans alone and found to have significantly lower pathogen colonization (Fig. 5 A and D) as measured by smaller lesion sizes (Fig. 5 B and E) and fewer sporangia recovered from the leaf surface (Fig. 5 C and F) compared with the cMYC-GFP or empty vector controls, respectively. This confirms the reduction in plant susceptibility to P. infestans observed transiently (Fig. 4). We propose that the reduction in susceptibility is due to AtPUB33 not being targeted by the effector and complementing loss of StUUBK activity (the potato protein targeted by Pi06087), hence overcoming the impact of the effector. It is possible that the reduction in susceptibility could have been due to overexpression of AtPUB33 per se, and indeed, we did not generate transgenic lines overexpressing StUUBK for direct comparison. However, we used transient expression experiments to demonstrate that AtPUB33 specifically undermines Pi06087 effector function. Transient expression of Pi06087 in transgenic N. benthamiana plants expressing GFP led to enhanced P. infestans colonization. This increase in pathogen colonization was not observed on plants expressing AtPUB33 (SI Appendix, Fig. S14). In contrast, expression of control effector F04089 enhanced P. infestans colonization on both GFP- and AtPUB33-expressing plants (SI Appendix, Fig. S14). These data indicate that expression of the cAtOrth AtPUB33 in host plants specifically undermines the virulence function of the effector, Pi06087, consistent with the effector failing to interact with and thus, manipulate AtPUB33. This provides support for the hypothesis that plant immunity can be enhanced through overexpression of nonhost orthologous proteins that escape effector manipulation.

Discussion

Many Effectors Fail to Enhance Susceptibility in Nonhost Plants. We selected RxLR effectors that enhance susceptibility when expressed in host plants—15 P. infestans effectors (28) and 10 H. arabidopsidis effectors (ref. 19 and this work)—and tested whether they would also enhance susceptibility in nonhost plants. Only three H. arabidopsidis effectors enhanced P. infestans colonization in N. benthamiana, whereas no P. infestans effectors enhanced H. arabidopsidis colonization in Arabidopsis (Fig. 1). Likely explanations for these failures are 1) that the effectors are unable to suppress immunity in the nonhost plants or 2) that the requirements for susceptibility differ for these two oomycete pathogens, one of which is a hemibiotrophic and the other of which is an obligate biotrophic. A third explanation, that the effectors are recognized by resistance proteins and, thus, trigger ETI, is unlikely as their expression did not trigger cell death in the nonhost plants.

A previous study demonstrated that the very few RxLR effectors conserved at the protein sequence level between H. arabidopsidis and Phytophthora species can suppress PTI and ETI in distantly related nonhost plants (30, 31). However, most H. arabidopsidis effectors are not conserved with Phytophthora species (7), and that was the case for those tested in Fig. 1. Both hypotheses to explain the failures of most of these effectors to enhance colonization in the nonhost plants are consistent with either the independent evolution of effectors with different roles in the two pathogens or the significant divergence of effectors from a common starting point. Nevertheless, three HaRxLs did enhance P. infestans colonization of N. benthamiana, suggesting that they function in this nonhost plant, albeit they were significantly less proficient at doing so compared with most PiRxLRs tested. This is perhaps indicative of effectors that only poorly interact with or are less efficient in appropriately manipulating orthologs of their targets in the nonhost. The effector HaRxL21, which significantly enhances P. infestans colonization of N. benthamiana (Fig. 1), targets the transcriptional repressor TOPLESS in the host Arabidopsis (47), raising the possibility that it is also capable of targeting this host protein in N. benthamiana. Interestingly, effector HaRxL44 interacts with the Arabidopsis mediator subunit med19a (20), which was confirmed here (Dataset S3), but failed to enhance P. infestans colonization, raising the possibility that it also fails to appropriately manipulate med19a in N. benthamiana. In contrast to the H. arabidopsidis effectors, no P. infestans effectors enhanced H. arabidopsidis colonization of Arabidopsis across all transgenic lines tested. Indeed, expression of two effectors, Pi04089 and Pi01654, consistently reduced colonization by H. arabidopsidis. Whether this is due to triggering resistance or failing to provide a metabolic change required for susceptibility remains to be tested. Interestingly, however, Pi04089 accelerated flowering time (SI Appendix, Fig. S3), perhaps suggesting 1) that it does indeed target an Arabidopsis protein but that this is an off-target interaction; 2) that it fails to appropriately manipulate its target; 3) that Arabidopsis and potato differ in their regulatory networks controlling immunity; or 4) that H. arabidopsidis and P. infestans have different requirements for susceptibility. In contrast to the results here, most tested candidate effectors from the poplar rust fungus Melampora larici-populina enhanced H. arabidopsidis colonization when expressed in Arabidopsis (48). This suggests that these M. larici-populina effectors are able to interact with and appropriately manipulate their targets in Arabidopsis. In the case of M. larici-populina, the pathogen lifestyle is very similar to H. arabidopsidis, in that both are obligate biotrophs, so defense responses to each may also be similar.

P. infestans RxLRs Interact with Diverse Host Proteins and Target Hubs Shared with Other Pathogens. Sixty-four previously described PiRxLRs (Dataset S1) (28) were screened in a
cY2H library derived from RNA extracted from potato–
P. infestans interactions (32). Twenty-four effectors revealed no
interacting proteins, potentially indicating that their targets are
not proteins; are proteins associated with host membranes and,
thus, interactions cannot be demonstrated in Y2H; or are pro-
teins, or regions of proteins, that are not represented in the
cY2H library (Fig. 2 and Dataset S2). The remaining 40
PiRxLRs interacted with 169 host proteins in the cY2H screens.
Some host proteins were represented by multiple yeast clones
emerging from a screen. All such interactions that have been
examined in more detail have been veri
fi
ed in planta using coIP
and/or bimolecular
fluorescence complementation (32–44).

Moreover, interactions between effector Pi06099 and StPhyB and
between effector Pi21388 and StCML36 were also verified in
planta here (SI Appendix, Figs. S7 and S8). The cY2H screens
thus offer many high-confidence candidate target proteins and
processes that are potentially manipulated by
P. infestans
effectors
in its host potato during infection, and thus, they provide a valu-
able resource to the research community.

The candidate targets of the 40 PiRxLRs represent proteins
involved in several biochemical processes, including phosphory-
lation or dephosphorylation, ubiquitination, DNA or RNA
binding, lipid binding, protein binding, and various enzymatic
activities (Dataset S2). A range of highly diverse host proteins
and processes are thus implicated as targets for manipulation
during late blight disease. Very few candidate membrane recep-
tor proteins associated with signal perception were observed,
potentially because interactions with such proteins would be

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<td>AtCAP2, Ca-dependent protein kinase adaptor</td>
<td>Pi15287, Pi04339, Pi07387</td>
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<td>Pi06087</td>
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PNAS 2022 Vol. 119 No. 35 e2114064119 https://doi.org/10.1073/pnas.2114064119 7 of 12
unlikely to occur in Y2H. Interestingly, not many candidate targets were revealed that are associated in previous studies with immunity. Those that were included signal transduction components, such as the mitogen-activated protein (MAP) kinase kinase MAP3K2 (44), MAP3Kε (37), and MAP3K5 (49) and transcriptional regulators, such as MYC2 (50) and Med19a (20).

Analysis of GO terms for biological processes among the AtOrth candidates (Dataset S3) revealed enrichment of 12 processes (SI Appendix, Fig. S9), including Golgi to plasma membrane transport and vesicle-mediated transport to plasma membrane. A recent report of proteomic data generated following immunoprecipitation of P. infestans effectors expressed in N. benthamiana (46) revealed enrichment of secreted proteins and proteins involved in vesicle trafficking. Our observations agree that secretion and subcellular trafficking are likely RXLR effector targets in the host.

Most P. infestans effectors (48) interact with more than 2 host proteins, with the maximum number being 18. In detailed studies, it has been shown that effectors, such as Avr-Piz-t from Magnaporthe oryzae, interact with functionally diverse proteins, such as the plasma membrane potassium channel OsAKT1 (51), the ubiquitin E3 ligases APIP6 (52) and APIP10 (53), the basic leucine zipper (bZIP) transcription factor APIP5 (54), and the nucleoporin-like protein APIP12 (55), suggesting multiple functions. Indeed, Avr3a from P. infestans has been reported to interact with the ubiquitin E3 ligase CMPG1 (32) and to associate in planta with dynamin-related protein 2 (56) and AtCAD7 (57). Multifunctional effectors are also a feature of bacterial type III secretion system arsenals (15), indicating that effectors can potentially play multiple virulence roles. In addition, we saw intraspecies convergence of multiple effectors on common host proteins. This was previously described by Weßling et al. (14), where it was shown that the extent of convergence correlates with the frequency of immune phenotypes for the respective genetic null mutants. Thus, while it is possible that some of these are false-positive interactions, the interaction topology has been observed before and is likely related to the biology of host–microbe interactions.

It is interesting to note that some candidate targets of PiRxLR effectors in potato are common targets of other pathogens, some of which are also regarded as highly interconnected regulatory hubs, such as CSN5 and PRA1. Other common targets include the endoplasmic reticulum associated transcription factor NTL9, LSU2, SEC5, importin-α, kinesin, SINA2, HSP23, Ftn2, Med19a, and 14-3-3 proteins (Dataset S4) (13, 14). While this interspecies convergence was described by Weßling et al. (14), our data suggest that similar host proteins are relevant for related pathogens in different plant species and hence, that the molecular pathogen–host interface is similar not only for different pathogens targeting the same host but also, for related pathogens targeting different hosts.

The Candidate Ortholog Y2H Screen. In the MoY2H screen, most PiRxLRs failed to interact with the cAtOrths of their potato cY2H interactors (Fig. 3 and Dataset S3). This suggests that in many cases, sequences of the putative orthologs had diverged sufficiently to abolish recognition due to the evolutionary distance between potato and Arabidopsis. Although the two Y2H screens differ in that the MoY2H system comprised FL candidate AtOrth sequences, whereas the potato cY2H library yielded a mixture of FL and truncated cDNAs, ~60% of potato cDNAs emerging from the cY2H screen were FL or almost so (Dataset S3), and we have observed that cloning FL versions of truncated sequences emerging from the cY2H screen produces similar interaction results (SI Appendix, Figs. S11 and S13) (34, 37, 39, 40, 43, 44). Therefore, failure of so many effectors to interact with cAtOrths may help to explain why P. infestans is unable to colonize Arabidopsis and provides support for the model proposed by Schulze-Lefert and Panstruga (5), which proposes that many effectors may fail to manipulate their targets in distant nonhost plants presumably due to sequence divergence, at least in regions of interaction with the effectors.

The four distinct interaction categories of cAtOrths uncovered in the ortholog screen suggest several possibilities. Category 1 cAtOrths, where PiRxLR effectors interact but there were no interactions with H. arabidopsidis effectors, suggests that although targets are sufficiently conserved at the protein level, they may not play a role in regulating immunity to H. arabidopsidis or effectors from H. arabidopsidis may target alternative proteins to alter the same host processes. Category 2 cAtOrths, which interacted with effectors from both pathogens, could indicate that there is conservation of both of protein structure and of infection strategies employed by both pathogens. Category 3 cAtOrths, where PiRxLRs failed to interact but H. arabidopsidis effectors did, also support the common infection strategies hypothesis. In fact, categories 2 and 3 revealed that a disproportionately large percentage of the cAtOrths interacts with H. arabidopsidis RxLRs, which is statistically significant when compared with a random selection of Arabidopsis proteins. Indeed, this screen has identified many proteins that interact with H. arabidopsidis effectors with no previously known roles in immunity. In contrast, the large number of cAtOrths belonging to category 4, where there was no interaction with effectors from either pathogen, underlines the evolutionary distance between the two pathosystems and may suggest either that the regulatory systems controlling immunity differ between the two plants or that the pathogens also employ different strategies for immune suppression that better suit their different infection cycles. For example, H. arabidopsidis is an obligate biotroph that relies on keeping its host alive to complete its life cycle. In contrast, hemibiotrophic pathogens, such as P. infestans, maintain living host cells for a shorter period before switching to a necrotrophic phase in which host cells may be actively killed. However, infection by the obligate biotroph Albugo allows colonization of Arabidopsis by P. infestans, thus breaking NHR, whereas H. arabidopsidis does not allow this (25). Interestingly, Albugo employs a different infection strategy to H. arabidopsidis in that it is more tolerant of certain Arabidopsis immune responses and better able to colonize these plants under stress conditions (58).
None of the *Arabidopsis* lines expressing *P. infestans* RxLRs consistently enhanced *H. arabidopsidis* infection to statistically significant levels (Fig. 1) whether they maintained interactions with candidate cAtOrths (Pi02860, Pi04314, Pi06099, Pi07387, Pi07555, Pi10654, Pi22798, and Pi22922) or failed to interact with them (Pi04089, Pi06087, Pi09218, Pi09585, Pi13628, Pi17316, and Pi22926) (Dataset S3). Potato interactors of Pi02860 (StNRL1) and Pi04314 (PIPlc isoforms) are verified targets of these effectors (39, 40). Failure of these effector transgenic lines to enhance *H. arabidopsidis* colonization may suggest that these targets are inappropriately manipulated in *Arabidopsis* or that their manipulation is not productive for *H. arabidopsidis* infection. In contrast, failure of Pi06087 (SF13), Pi09858 (SF14), and Pi13628 (SF15) to interact with cAtOrths is consistent with the previously reported inability of these effectors to suppress PTI in *Arabidopsis* (59).

Of course, not all cAtOrths were cloned and tested, and many effectors interacted with multiple targets, where some interactions were maintained but others were not. However, there are examples where PiRxLR expression significantly reduced *H. arabidopsidis* colonization of *Arabidopsis* (Pi04089 and Pi10654), suggesting that these effectors exhibit an activity whether they interact with the cAtOrth of their host potato interactor or not. Pi04089 is interesting as the transgenic lines also had an earlier flowering phenotype, and although Pi04089 did not interact with At5g15270 (SI Appendix, Fig. S11), several closely related family members of this RNA binding protein in *Arabidopsis* are known to regulate flowering time (60), suggesting an off-target action of the effector. In this regard, it is interesting to note that whereas KRPB1 acts as a susceptibility factor (33), its closely related homolog FLK is a positive regulator of flowering (61), and a recent preliminary report suggests that it is also a positive regulator of plant immunity (62). It will be interesting to see whether Pi04089 interacts with a closely related K homology (KH) RNA binding protein, such as FLK. In contrast, whereas the interaction is maintained between Pi10654 and AtDegP, failure of transgenic lines expressing this effector to enhance pathogen colonization suggests that the effector is not able to correctly manipulate the cAtOrth, that manipulation differentially regulates immunity between *Arabidopsis* and potato, or that the *H. arabidopsidis* infection process does not have the same requirements as that of *P. infestans*. Indeed, there are many examples where mutation of effector targets can have both an enhanced disease resistance or enhanced disease susceptibility phenotypes in response to challenge with different pathogens or even different strains of the same pathogen (14).

**Expression of Nonhost Targets in Host Plants.** Historically, engineering disease resistance has often involved transfer of *R* genes from one plant species to another (63, 64). Transfers typically make a huge impact on defense by enhancing the recognition specificity but can be overcome by rapidly evolving pathogens through mutation or loss of the recognized effector. Pyramiding multiple *R* genes is expected to enhance the durability of such resistance. Increases of recognition specificity can also be engineered through the transfer of pathogen-associated molecular pattern receptors, such as the EF-Tu (elongation factor thermo unstable) receptor to new species to help combat disease (65). Other approaches to enhance immunity involve the mutagenesis or knockdown of so-called *S* genes, which are required by the pathogen for a successful colonization of its host (63, 66, 67). Here, we provide proof of concept for an approach to enhance disease resistance by transfer of effector target proteins from nonhost to host plants. Expression of AtPUB33, a nonhost *Arabidopsis* ortholog of the effector target StUBK (34), increased resistance to *P. infestans* in two different host species, potato and *N. benthamiana* (Fig. 5). As AtPUB33 failed to interact with the PiRxLR effector Pi06087/SF13 (SI Appendix, Fig. S12), it presumably is not targeted by *P. infestans* for manipulation by this effector. Pi06087 did not enhance pathogen colonization on transgenic plants expressing AtPUB33 (SI Appendix, Fig. S14). This result indicates that although effector Pi06087 can target potato StUBK and presumably prevent its function, it is unable to target AtPUB33, leaving the nonhost ortholog able to effectively complement for the effector-mediated loss of StUBK activity. Although the level of enhanced resistance was modest compared with *R* gene introgression, pyramiding could incorporate multiple nonhost genes with additive effects on disease resistance through escaping pathogen effector manipulation. It may be possible to use RNA editing/CRISPR to mutate discreet effector-interacting regions of host effector target proteins to resemble the nonhost variant. Identifying nonhost effector target orthologs that evade manipulation could provide a strategy to promote durable disease resistance.

**Materials and Methods**

**Plant Growth.** *N. benthamiana* was grown at 22 °C in 16-h days and 8-h nights at 18 °C. Ambient light was maintained between 200 and 450 W/m². A. thaliana was grown at 20 °C with 12-h day length.

**Effector Cloning.** *H. arabidopsidis* RxLR candidates were amplified from cDNA from spores and infection. The *P. infestans* effector collection was generated as described (28). *H. arabidopsidis* and *P. infestans* candidate effectors were cloned minus the signal peptide (Dataset S1). Dataset S1 shows primer sequences for the addition of attachment site B (ATTB) recombination sites by nested PCR. Recombination of attB-effector PCR products with pDonrZeo or pDonr201 was performed to generate Gateway entry clones. Effectors were recombined into pB7WG2 (68) or pEG201 (69) destination vectors and transferred into Agrobacterium to conduct transient assays or make transgenic *Arabidopsis*.

**Generation of Arabidopsis Transgenics.** *Arabidopsis* ecotype Col-0 was dipped (70) with Agrobacterium harboring PiRxLRs cloned into pEG201 (69) expressed with a Cauliflower mosaic virus (CaMV 35S) promoter. Lines were selected on Basta soaked soil (1 mL/L) until homozygosity at T3. Three independent lines were generated for each effector. Expression of *P. infestans* effector messenger ribonucleic acid (mRNA) in *Arabidopsis* transgenics was verified using RT-PCR. RNA was extracted from pooled 14-d-old seedlings using a RNeasy Plant Mini Kit according to the manufacturer but with the addition of deoxyribonuclease (DNase) treatment using a Qiagen RNase-Free DNase Set. cDNA was synthesized using SuperScript II reverse transcriptase. PCR was performed using Bioline BioMix Red. Dataset S6 shows primer sequences.

**H. arabidopsidis Infection Assays.** Infections with *H. arabidopsidis* isolate Noks1 were performed on 2-wk-old seedlings as described (71). Noks1 was maintained on 7-d-old *Arabidopsis* ecotype Col-0 seedlings. Spores were harvested from infected Col-0 seedlings, filtered through miracloth, and adjusted to 30,000 spores/mL. Sporangiophores per seedling were counted 4 days post-infection (dpi) using a dissecting microscope (15 plants per pot, three pots per tray, two replicates with at least two lines per transgenic).

**P. infestans Culture and Infection Assays.** Sporangia were prepared from *P. infestans* strain 88069 after growth at 19 °C on Rye agar plates for 11 to 14 d. Sporangia were harvested (72) to a concentration of 50,000/mL in sterile distilled water (SDW). Leaves (three per plant; greater than or equal to six plants per replicate; less than or equal to three replicates) of transgenic *Arabidopsis* were inoculated with 10 µL of *P. infestans* inoculum. Lesions were measured at 7 dpi. Sporulation was harvested from leaves in 3 mL of sterile water and counted using a counting chamber.

**Agrobacterium-Mediated Transient Infection Assays.** Agrobacterium strains GV3101 or AGL1 expressing PiRxLR or cAtOrth constructs were grown at 28 °C.
Overview of experimental procedures:

- **Ortholog Identification and Cloning.** cAtOrths of potato Y2H interactors were cloned by PCR and Sanger sequencing. Isolated pDest-DB clones were transformed into Gateway entry vectors. cAtOrth sequences were generated for each bait construct and transformed individually with a domain containing pDest-DB vectors, and recombinants were verified by Gateway recombinant cloning in pDEST-AD and verified by PCR and Sanger sequencing (Dataset S5).

Convergence Analysis. We conservatively model that 10,000 different proteins are represented in the potato cY2H library. To estimate the significance of convergence, we randomly sampled (n = 215) interactions with 10,000 available proteins (random sampling with replacement). In each iteration, the number of distinctly targeted proteins was counted, and the random density distribution was plotted and compared with the number of experimentally observed distinct targets (n = 169). To ensure robustness, we repeated the analysis with smaller search spaces of 5,000 and 2,000 proteins, resulting in the same conclusion.

Arabidopsis Candidate MoY2H Screens. The Y2H assay was performed as described (12) with minor modifications. Open reading frames (ORFs) coding for H. arabidopsidis and P. infestans effector candidates were transferred into DNA-binding domain (DB) containing pDest-DB vectors, and recombinants were confirmed by PCR and Sanger sequencing. Isolated pDest-DB clones were transformed into Saccharomyces cerevisiae Y8930 (mating type α) by lithium-acetate transformation. Positive transformants were selected on medium lacking leucine, and archival stocks were prepared and stored at ~80°C. cAtOrths were cloned by Gateway recombinant cloning in pDEST-AD and verified by PCR and Sanger sequencing (Dataset S5). pDEST-AD clones containing cAtOrths were transformed into Arabidopsis thaliana (Col-0 ecotype) and transgenic lines were generated by Agrobacterium-mediated transformation.

Potato Y2H Screens. Screens were conducted with the Invitrogen ProQuest Potato Y2H Screens. Candidate effector targets were identified by yeast two-hybrid screens. To test for prey autoactivation, a prey vector (EV) control was used. The cAtOrth library was evaluated for autoactivation by one-way ANOVA or Kruskal–Wallis one-way ANOVA on ranks with multiple comparisons vs. the control group using the Holm–Sidak method. ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05.

Transgenic plants overexpressing AtPub33 show increased resistance to P. infestans. (A) The box plot shows P. infestans lesion diameters in five independent transgene generation 2 (T2) N. benthamiana lines expressing cMYC-AtPub33 compared with a T2 cMYC-GFP control. (B) The box plot shows P. infestans sporangia recovered per milliliter in five independent T2 N. benthamiana lines expressing cMYC-AtPub33 compared with a T2 cMYC-GFP control. (C) Representative leaf images showing P. infestans lesions on five independent potato transgenic lines expressing untagged AtPub33 compared with an empty vector (EV) control. (D) The box plot shows P. infestans sporangia recovered per milliliter in five independent potato transgenic lines expressing untagged AtPub33 compared with an EV control. (E) Representative leaf images showing P. infestans lesions on five independent potato transgenic lines expressing untagged AtPub33 compared with an EV control. (F) Representative leaf images showing P. infestans lesions on five independent potato transgenic lines expressing untagged AtPub33 compared with an EV control.
in S. cerevisiae Y8800 (mating type a) by lithium-acetate transformation, and positive transformants were selected and stored in 40% glycerol at −80 °C. Autoactivator removal was performed as described (14). For the primary Y2H screen, pDest-AD-Ortholog clones were grown on synthetic complete solid medium lacking tryptophan for 2 d, and pools of 75 individuals were generated. Uniform distribution of clones was checked as described (12). Single DB-effector clones were mated with pools of 75 AD-CAOrth clones. Five microliters of freshly grown DB and AD yeast was spotted on top of each other on yeast extract peptide dextrose growth medium (YPD) using a liquid handling robot. Identification of interacting effector-ortholog clones was as described (12, 13, 73). The screen was repeated once. Interactions were verified when they were positive in three of four repeated matings and autoactivation was not detected. Methods to define effector ortholog–protein interactions were as described (13, 73). Consequently, key parameters of the interactome screen, such as sampling and assay sensitivity, are identical between experiments, and integration of data will not introduce bias due to the experimental design (74).

**Common Targets between H. arabidopsidis and P. infestans Effectors.** To assess whether H. arabidopsidis and P. infestans effectors have more common Arabidopsis targets than expected, the number of distinct targets in both screens was compared with 10,000 random picks from the Arabidopsis Interactome version 1 “main screen” (AI-1 MAIN) (73), previously used for effector-host interaction screening. To model the expectation of finding H. arabidopsidis effectors by unbiased screening, we randomly picked 10,000 times 116 target proteins (i.e., the number of P. infestans–potato target orthologs that we tested for interactions with H. arabidopsidis effectors) and counted the number of real H. arabidopsidis effector targets observed previously (13, 73). Comparing the observed value of 10 H. arabidopsidis effector interactions with P. infestans effector target orthologs with random distribution shows a significantly higher rate of interaction detection using the ortholog approach than using unbiased screening.

**Generation of N. benthamiana Transgenics.** Approximately 40 small leaf disks per construct of N. benthamiana leaves agroinfiltrated with CMSV-35S-driven expression of myc-GFP or myc-AtUBP33 (OD600 = 0.05) were harvested at 2 dpi and surface sterilized in 2% bleach with 1 drop of Tween20 per 50 mL for 10 min. Leaf disks were washed five times in SDW and aseptically transferred to shoot-inducing media plates (Murashige and Skoog medium, MS, 2% sucrose, 0.8% agar, 0.5 mg/L 1-Naphthalene-acetic acid (NAA), 200 μg/mL timentin, 50 μg/mL kanamycin). Plates were renewed every 10 d for 2 to 3 mo until shoots appeared. Shoots were then transferred to root-inducing media (MS, 2% sucrose, 0.8% agar, 0.5 mg/L NAA, 200 μg/mL timentin, 50 μg/mL kanamycin). On rooting, plantlets were transferred to soil. Positive transformants were confirmed by immunoblot; five individual lines per construct with detectable protein expression were recovered. Seeds collected from T0 and T1 plants were sown on MS supplemented with kanamycin selection, and transgene expression was confirmed by immunoblots.

**Generation of Potato Transgenics.** Transgenic potatoes expressing an untagged form of AtUBP33 under a CaMV 35S promoter and nopaline synthase (Nos) terminator were made by Simplot Plant Sciences (J. R. Simplot Company) as described (75), except that kanamycin was used as a selectable marker.

**Immunoblotting.** Transgenic N. benthamiana plant lines or protein fusions transiently overexpressed at 2 dpi in N. benthamiana were tested by immunoblotting to assess protein presence and stability. Proteins were extracted using GTEN extraction buffer (10% glycerol; 25 mM Tris, pH 7.5; 1 mM Ethylenediaminetetraacetic acid (EDTA); 150 mM NaCl; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM dithiothreitol (DTT); 0.5% Nonidet P-40; protease inhibitor tablet) mixed with 2× sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, loaded onto 12% SDS-PAGE gels, and run for 2 h at 120 V. Gels were blotted with nitrocellulose membrane for 1.5 h at 30 V with Ponceau staining to demonstrate transfer and loading. Membranes were blotted in 5% milk in 1× PBST (137 mM NaCl; 12 mM phosphate; 2.7 mM KCl, pH 7.4; 0.2% Tween20) for 1 to 2 h before the addition of primary antibodies overnight.

coIP. Protein fusion constructs were transiently overexpressed in N. benthamiana using Agrobacterium-mediated expression. Leaf disks were collected 48 h after infiltration. Samples were ground in liquid nitrogen, and tissue was resuspended in 500 μL GTEN extraction buffer (as above), gently vortexed, placed on ice for 10 min, and centrifuged at 13,000 × g for 10 min at 4 °C. For input samples, 40 μL of sample was removed, mixed with 40 μL 2× SDS-PAGE sample buffer, boiled at 95 °C for 10 min, and stored at −20 °C for western blot analysis. The remaining sample extract was incubated with 20 μL GFP-Trap M magnetic beads (Chromotek); beads were washed three times with 500 μL ice cold wash buffer (GTEN with 1 mM PMSF) on a rotary mixer for 1 h at 4 °C. Beads were magnetically separated from the sample supernatant and washed three times with 500 μL ice cold wash buffer; then, they were resuspended in 50 μL 2× SDS-PAGE sample buffer and boiled at 95 °C for 10 min. The resulting samples were separated by SDS-PAGE and analyzed by immunoblotting as above.

**Confocal Imaging.** Leaf cells from N. benthamiana were imaged as described (72) at 2 dpi using a Zeiss 710 microscope with Zeiss PL APO 403/1.0 water dipping objectives. GFP was excited at 488 nm with an argon laser, and emissions were collected at 500 to 530 nm. Monomeric red fluorescent protein (mRFP) was excited using a 561-nm line diode laser with emissions collected at 600 to 630 nm. The pinhole was set at 1 airy unit. Single optical slices and z stacks were collected from cells expressing low levels of protein fusions to minimize potential artifacts. Images were processed using the Zen 2010 software.

**Supplemental Information.** SI Appendix includes SI Appendix, Figs. S1–S14 and Dataset S1–S6.

**Data, Materials, and Software Availability.** All study data are included in the article and/or supporting information.

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The authors declare no competing interest.

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