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 orthologues (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 PiSF13, 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-nonhost target interaction, transferring effector target orthologues from non-host to host plants is a strategy to reduce disease.

host range | plant immunity | effector-triggered susceptibility | oomycete | plant–microbe interactions

Plant pathogenic microorganisms infect their hosts by secretion of effector proteins that can act outside (apoplastic effectors) or within (cytoplasmic effectors) plant cells to suppress immunity (1, 2). Yet, most pathogenic microbes equipped with effectors are unable to infect the majority of plant species. Nonhost resistance (NHR) is the phenomenon whereby all genotypes of a particular plant species are resistant to all genotypes of a given pathogen species, and as such, it is expected to be both broad spectrum and durable (3). In general, NHR is thought to result from one or more of the following factors: 1) the existence of preformed physical or chemical barriers preventing the pathogen getting a foothold in the plant; 2) recognition of pathogen effector proteins by plant resistance (R) proteins, leading to strong effector-triggered immunity (ETI); or 3) the failure of pathogen effectors to suppress pathogen-associated molecular pattern–triggered immunity (PTI) (4). If one solely takes into account inducible plant immunity, a model proposed by Schulze-Lefert and Panstruga (5) suggests that evolutionary distance may influence the relative contributions of PTI and ETI to NHR. Their model proposes that in nonhost plants that are closely related to the host, pathogen effectors are predicted to retain their interactions and activities upon target proteins that are likely to be closely related at the sequence level to those in the host plant. Under such circumstances, failure of the pathogen to infect is more likely to be due to ETI (i.e., the recognition of effectors by conserved R proteins). Conversely, in plants more distantly related to the normal host of a pathogen, failure to colonize is more likely to be due to lack of PTI suppression. This could be due to an inability of pathogen effectors to interact with and/or correctly manipulate their evolutionarily less conserved targets, rendering them unable to suppress PTI, which restricts pathogen colonization (5, 6).

In the last decade, strides have been made in both the identification of pathogen effector repertoires through genome/transcriptome sequencing and searches for conserved motifs (7–10) and their cognate plant target proteins using various protein–protein interaction...
and sidis and distantly related nonhost pathosystems. RxLR (Arg
ful. In contrast, with only where plant defenses have been suppressed by preinfection (23) but not the model plant
plant, and the model solanaceous plant
during host colonization, several of which have been functionally
H. arabidopsidis
exploit NHR for crop protection. Some oomycete effectors have evolved to use the activity of their
oomycete cytoplasmic effectors target diverse pathways and pro-
(MAPKs) (16). Many of the fungal effectors whose mode of
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 compo-
nents, 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 pro-
cesses 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 suscepti-
(bility) (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, egg-
plant, and the model solanaceous plant Nicotiana benthamiana (23) but not the model plant Arabidopsis, a member of the Brassi-
ceae (24). Arabidopsis can be artificially made to allow P. infes-
tans to complete its infection cycle in laboratory conditions but
only where plant defenses have been suppressed by preinfection with
Allbugo laibachii (25, 26). Attempts to render Arabidopsis
susceptible to P. infestans by genetic means have not been success-
ful. 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. infes-
tans and H. arabidopsidis, respectively, will unlock potential to
exploit NHR for crop protection.

In this study, we expressed RxLR effectors from H. arabidop-
sidis 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 Arabi-
dopsis 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 Arabi-
dopsis, 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 effect-
ors contribute to pathogenicity in both host and nonhost pathos-
systems, 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. arabi-
dopsidis on Arabidopsis). Fifteen PiRxLR effectors were selected on
the basis that they had been previously to enhance P. infes-
tans colonization (28). They were expressed transiently (with
green fluorescent protein (GFP) tags) 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–effectors fusion protein was stable in planta (SI Appendix,
Fig. S1A). We then expressed 10 HaRxLs in N. benthamiana. In
counter to the PiRxLRs, only three HaRxLs significantly enhanced P. infestans colonization of N. benthamiana compared
with the GFP control, and those that reproducibly enhanced colo-
nization 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. arabi-
dopsidis. At least two transgenic lines expressing each H. arabi-
dopsidis effector were selected and shown to be significantly
more susceptible to H. arabidopsidis infection as measured by
sporangiophere counts, compared with control plants (Fig. 1C). In
contrast, when transgenic Arabidopsis were generated express-
ing the 15 RxLR effectors from the nonhost pathogen P. infes-
tans (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 coloniza-
tion, suggesting that activity of these effectors was detrimental to
H. arabidopsidis infection. In order to understand this, we inves-
tigated whether these lines had an altered developmental pheno-
type. 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 × 10⁶) 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 Pi1PI04 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...
PiRxLR effectors maintain interactions with candidate orthologs of their targets in a distinctly 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 cAtOrths 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 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 orthology of their targets in a distinctly 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 cAtOrths 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).

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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...

Fig. 2. PiRxLR cY2H interaction network in potato. (A) Network diagram representation of the cY2H interaction network of 64 PiRxLR effectors (yellow circles) with 169 potato candidate target proteins (green circles). Straight edges indicate 215 protein-protein interactions. (B) Histogram showing the distribution of counts of PiRxLR effectors interacting with a given number of candidate target proteins (equivalent to the degree for each PiRxLR node in A). (C) Histogram showing the distribution of counts of candidate potato target proteins interacting with a given number of PiRxLR effectors (equivalent to the degree for each candidate target protein in A).
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-isofoms (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 effector 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 Weißling 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 effectors, significantly altered P. infestans infection levels (Fig. 4). AT5G15270 overexpression enhanced P. infestans colonization compared with the control. Interestingly the potato equivalent, StKRBP1, acts as an S factor, and its overexpression also boosts P. infestans colonization of N. benthamiana (33). Although
At5g15270 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 communoprecipitate At5g15270 (SI Appendix, Fig. S11D). Moreover, StKRBP1 and At5g15270 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 predicted potato ortholog of AtPUB33, called StUBK (PGSC0003DMT400000116), is targeted by the P. infestans effector Pi06087 (PiSRF1/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 StUBK 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 StUBK 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 StUBK 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 Pi04089 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 hemibiotroph and the other of which is an obligate biotroph. 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 Pi10654, 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 Melampsora 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 proteins, 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 verified 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 valuable resource to the research community.

The candidate targets of the 40 PiRxLRs represent proteins involved in several biochemical processes, including phosphorylation 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 receptor proteins associated with signal perception were observed, potentially because interactions with such proteins would be

### Table 1. cAtOrths selected for expression in *N. benthamiana*

<table>
<thead>
<tr>
<th>cAtOrths</th>
<th>Arabidopsis annotation</th>
<th>PiRXLR interacting in potato cY2H</th>
<th>Interacts in MoY2H with PiRxLRs</th>
<th>Interacts in MoY2H with HpaRxLRs</th>
<th>Interaction category of cAtOrth</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G14000</td>
<td>AtVIK, VH1-interacting kinase</td>
<td>Pi17309, Pi17316</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT1G34190</td>
<td>NAC domain containing protein 17</td>
<td>Pi03192</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>AT1G71230</td>
<td>Encodes a subunit of the COP9 complex</td>
<td>Pi07555, Pi13625, Pi13959</td>
<td>Yes</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>AT2G17990</td>
<td>AtCAP2, Ca-dependent protein kinase adaptor</td>
<td>Pi15287, Pi04339, Pi07387</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>AT2G45910</td>
<td>AtUPB33, UBOX and kinase domain protein</td>
<td>Pi06087</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT2G47940</td>
<td>AtDegP2 protease</td>
<td>Pi10654</td>
<td>Yes</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>AT3G06720</td>
<td>AtIMPA-1 importin-α</td>
<td>Pi22798</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>AT3G10190</td>
<td>AtCML36, calmodulin-like 36</td>
<td>Pi21388</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>AT3G15220</td>
<td>Protein kinase superfamily protein</td>
<td>Pi13628</td>
<td>No</td>
<td>No</td>
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</tr>
<tr>
<td>AT3G58040</td>
<td>Encodes an RING finger domain protein</td>
<td>Pi04339</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>AT4G08320</td>
<td>AtTPR8, tetratricopeptide repeat 8</td>
<td>Pi07689, Pi14371</td>
<td>No</td>
<td>Yes</td>
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</tr>
<tr>
<td>AT4G14880</td>
<td>O-acetylserine (thiol)lyase</td>
<td>Pi14371</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>AT4G16143</td>
<td>AtIMPA-2, importin-α-isofrom 2</td>
<td>Pi22798</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>AT4G20360</td>
<td>AtSVR11, suppressor of variegation 11</td>
<td>Pi12926</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>AT4G25200</td>
<td>AtHSP23.6-MITO, small heat-shock protein 23</td>
<td>Pi07689</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>AT4G27060</td>
<td>AtTORTIFOLIA1, microtubule-associated protein</td>
<td>Pi06308</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
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<tr>
<td>AT5G14720</td>
<td>Protein kinase superfamily protein</td>
<td>Pi11383</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT5G15270</td>
<td>RNA binding KH domain-containing protein</td>
<td>Pi04089</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT5G24590</td>
<td>AtTIP, TCV-interacting protein, AtNAC91</td>
<td>Pi09218</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT5G24660</td>
<td>AtLSU2, response to low sulfur 2</td>
<td>Pi15287</td>
<td>No</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>AT5G28770</td>
<td>bZIP protein BZO2H3</td>
<td>Pi07555</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>AT5G64370</td>
<td>AtPYD3 encodes a β-ureidopropionase</td>
<td>Pi15278</td>
<td>No</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>AT5G65430</td>
<td>ATM1N10, general regulatory factor 8</td>
<td>Pi02860</td>
<td>Yes</td>
<td>No</td>
<td>1</td>
</tr>
</tbody>
</table>
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 kinases MAP3Kβ2 (44), MAP3Ke (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 the plasma membrane potassium channel OsAKT1 (51), the plasmatic reticulum associated transcription factor NTL9, LSU2, nucleoporin-like protein APIP12 (55), suggesting multiple functions.

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 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 or effectors from H. arabidopsidis may target alternative proteins to alter the same host processes. Category 4 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).

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 Schulte-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.

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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 (PITc 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 (STF3), Pi09585 (STF14), and Pi13628 (STF5) 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 early 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 homolog (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. 3). 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 (ATIB) recombinase 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 pB7WG2F (68) or pEG201 (69) destination vectors and transfected 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 deoxynucleoseline (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 T-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 replicates; less than or equal to three replicates) of transgenic N. benthamiana and potato lines (three leaves per plant; four plants per replicate; two replicates) were drop inoculated with 10 μL of P. infestans inoculum. Lesions were measured at 7 dpi. Sporangia were 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.
determine the interacting plant protein, and clones were cotransformed into and test samples were in gone adjusted to an optical density (OD) at 600nm of 0.1. In addition, we used the EnsemblPlants (plants. potato cDNA activation domain system and yeast strain MaV203 according to the manufacturer. DNA binding baits were taken. Competent cells into MaV203 cells and recovered by nutritional selection, and subsequently, domain

Screens were conducted with the Invitrogen ProQuest (2-(N-morpholino)ethanesulfonic acid): 10 mM MgCl2 with 200 \\( \mu \)M acetoxyrisogone adjusted to an optical density (OD) at 600nm of 0.1. Agrobacterium control and test samples were infiltrated on either half of an N. benthamiana leaf (three per plant; greater than or equal to six plants per replicate; greater than or equal to three experimental replicates) before being drop inoculated 24 h later with 10 \\( \mu \)L P. infestans inoculum (50,000 sporangia/mL). Infection lesions were measured at 7 dpi, and Student’s t tests or Mann–Whitney rank sum tests were performed to determine statistical significance.

**Potato Y2H Screens.** Screens were conducted with the InVitrogen ProQuest system and yeast strain MaV203 according to the manufacturer. DNA binding domain “bait” fusions to each P. infestans effector were generated using Gateway recombination from relevant pDom201 clones. These were transformed into MaV203 cells and recovered by nutritional selection, and subsequently, they were tested to eliminate reporter gene autoactivation. Competent cells were generated for each bait construct and transformed individually with a potato cDNA activation domain “prey” cY2H library. Interacting clones were selected as described previously (72). Interacting clones were sequenced to determine the interacting plant protein, and clones were cotransfected into yeast to confirm interaction and tested for prey autoactivation.

**Ortholog Identification and Cloning.** cAtOrths of potato Y2H interactors were found by performing RBBH analysis between the two genomes. If no RBBH was found, the BBH was taken. In addition, we used the EnsemblPlants (plants.ensembl.org/info/genome/compara/homology_method.html) phylogenetic study of candidate orthologs as an independent assessment. The coding sequences of the cAtOrths were found to be present in the existing matrix Y2H Arabidopsis clone library (13, 14) or amplified by nested PCR to add ATB recombinase sites, and they were recombined into Gateway entry vectors. cAtOrth sequences and primers are shown in 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 transfected 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...
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 peptone dextrose growth medium (YEPD) 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” (Al-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 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 CaMV 35S-driven expression of myc-GFP or myc-AtPUB33 (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, 2 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L 1-Naphthaleenic-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 were transferred per 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 immunoblot.

Generation of Potato Transgenics. Transgenic potatoes expressing an untagged form of AtPUB33 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 p40; protease inhibitor tablet) mixed with 2x sodium dodecylsulfate polyacrylamide gel electrophoresis (SOS-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 blocked in 5% milk in 1× PBS (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:


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