Plant pathogen effector utilizes host susceptibility factor NRL1 to degrade the immune regulator SWAP70

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Plant pathogens deliver effectors into plant cells to suppress immunity. Whereas many effectors inactivate positive immune regulators, other effectors associate with negative regulators of immunity: so-called susceptibility (S) factors. Little is known about how pathoens exploit S factors to suppress immunity. Phytophthora infestans RXLR effector Pi02860 interacts with host protein NRL1, which is an S factor whose activity suppresses INF1-triggered cell death (ICD) and is required for late blight disease. We show that NRL1 interacts in yeast and in planta with a guanine nucleotide exchange factor called SWAP70. SWAP70 associates with endosomes and is a positive regulator of immunity. Virus-induced gene silencing of SWAP70 in Nicotiana benthamiana enhances P. infestans colonization and compromises ICD. In contrast, transient overexpression of SWAP70 reduces P. infestans infection and accelerates ICD. Expression of Pi02860 and NRL1, singly or in combination, results in proteasome-mediated degradation of SWAP70. Degradation of SWAP70 is prevented by silencing NRL1, or by mutation of Pi02860 to abolish its interaction with NRL1. NRL1 is a BTB-domain protein predicted to form the substrate adaptor component of a CULLIN3 ubiquitin E3 ligase. A dimerization-deficient mutant, NRL1MQ, fails to interact with SWAP70 but maintains its interaction with Pi02860. NRL1MQ acts as a dominant-negative mutant, preventing SWAP70 degradation in the presence of effector Pi02860, and reducing P. infestans infection. Critically, Pi02860 enhances the association between NRL1 and SWAP70 to promote proteasome-mediated degradation of the latter and, thus, suppress immunity. Preventing degradation of SWAP70 represents a strategy to combat late blight disease.

potato blight | effector-triggered susceptibility | disease resistance | pathogenicity | virulence

Plant innate immunity consists of two inducible layers that provide defense against microbial infections. The first of these, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), involves the detection of conserved microbial molecules, or the damage-associated products of microbial activity, by pattern-recognition receptors at the cell surface. Microbes successfully colonize plants by delivering effector proteins either to the inside (intracellular or cytoplasmic effectors) or outside (apoplastic effectors) of plant cells to attenuate PTI (1–5). The second inducible layer of immunity (effector-triggered immunity or ETI) involves the perception of effectors by cytoplasmic nucleotide-binding, leucine-rich repeat (NB-LRR) resistance (R) proteins (4). Effectors exploit a range of mechanisms to manipulate host immunity. Thus, identification of the targets of effectors in the host is essential to understand pathogen virulence and plant susceptibility. Many effector targets have been identified (1, 2, 5). The majority are positive regulators of immunity whose activity is suppressed as a consequence of effector interaction. In contrast, some targets either negatively regulate immunity or otherwise support a compatible interaction that benefits the pathogen. These targets in the host can be regarded as susceptibility (S) factors (6, 7).

Fungi and oomycetes are among the most damaging pathogens in agriculture. The oomycete Phytophthora infestans causes the devastating disease late blight on potato (Solanum tuberosum) (8, 9). P. infestans delivers so-called RXLR effectors into plant cells to modify host proteins to suppress immunity (5, 10). Some RXLR effectors target S factors. Examples include: the RXLR effector Pi04089, which interacts with a K-homology RNA-binding protein, KRBP1, to promote susceptibility (11); Pi04314/RD24, which interacts with three host isoforms of protein phosphatase 1 (PP1) catalytic subunits, forming unique holoenzymes that negatively regulate defense (12); and AVR2, which up-regulates a brassinosteroid-responsive basic helix-loop-helix transcription factor that suppresses immunity (13). Altering S-gene expression is a strategy particularly adopted and expanded by Xanthomonas species. For example, five Xanthomonas TAL effectors target the sugar transporter genes OsSWEET11 and OsSWEET14 that are S factors (14–16). Effectors from other bacteria (17–19) and nematodes (20, 21) have also been reported to target S factors. However, little is understood about precisely how S factors are exploited by pathogen effectors to undermine immunity.

In a previous study (22) we identified a host target of RXLR effector Pi02860-potato NPH3/RPT2-LIKE1 protein (NRL1), a predicted substrate adaptor component of a CULLIN3-associated ubiquitin E3 ligase. A dimerization-deficient mutant, NRL1MQ, fails to interact with SWAP70 but maintains its interaction with Pi02860. NRL1MQ acts as a dominant-negative mutant, preventing SWAP70 degradation in the presence of effector Pi02860, and reducing P. infestans infection. Critically, Pi02860 enhances the association between NRL1 and SWAP70 to promote proteasome-mediated degradation of the latter and, thus, suppress immunity. Preventing degradation of SWAP70 represents a strategy to combat late blight disease.

Significance

Plant pathogens, such as the infamous potato blight agent Phytophthora infestans, deliver effectors inside living plant cells to promote disease. Recent evidence suggests that some effectors will associate with endogenous negative regulators of immunity, or so-called susceptibility (S) factors, inside living plant cells. To date, little is known about how effectors exploit S factors to inactivate the immune system. We discovered that P. infestans effector Pi02860 uses the potato StNRL1 protein as an S factor by promoting its ability to target a positive regulator of immunity, StSWAP70, for proteasome-mediated degradation. Our results reveal that protecting StSWAP70 from degradation by reducing the activity or availability of NRL1 provides a means to attenuate late blight disease.

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two-hybrid (Y2H) screen that identified the guanine nucleotide exchange factor (GEF) SWAP70 as a candidate substrate of NRL1. SWAP70 acts as a positive regulator of immunity against P. infestans by promoting INF1-trigged cell death (ICD). We reveal that host protein NRL1 acts as an endogenous negative regulator of immunity by promoting proteasome-mediated turnover of SWAP70. Further analyses showed that Pi02860 promotes NRL1-mediated degradation of SWAP70. Loss of NRL1 by virus-induced gene silencing (VIGS) or expression of a dominant-negative dimerization-deficient NRL1 mutant in Nicotiana benthamiana prevented the degradation of SWAP70 by Pi02860. A mutation of Pi02860 that abolishes its interaction with NRL1 also prevent the degradation of SWAP70. Our results reveal how pathogens can exploit the endogenous negative regulators of immunity in the plant to promote disease.

Results

StNRL1 Forms Homodimers and Interacts with the Potato GEF StSWAP70. To explore the mechanism of Pi02860 action in plants, a Y2H screen was performed to identify candidate interactors of StNRL1. The Y2H library, made from cDNA of potato infected with P. infestans (23), was screened with a GAL4 DNA-binding domain–StNRL1 fusion (bait) construct to a depth of 4.6 × 10⁶ yeast cotransformants. Thirty-four yeast colonies were recovered from selection plates that contained GAL4 DNA-binding domain–StNRL1 fusion (bait) construct and StSWAP70 prey construct (prey) fusions. These included seven clones containing 14-3-3 proteins, and one clone each encoding a protein phosphatase 2A regulatory subunit and a HAT4-like homeobox-leucine zipper protein (SI Appendix, Table S1). In addition, 11 clones yielded sequences corresponding to a potato plekstrin homology-diffuse β lymphoma homology (PH-DH) domain protein (SI Appendix, Table S1). The protein is highly similar to SWAP70 from rice and Arabidopsis (SI Appendix, Fig. S1), and is thus hereafter referred to as StSWAP70 (S. tuberosum SWAP70). SWAP70, a GEF, was reported to play a positive role in regulating both PTI and ETI (24, 25). It is thus a potential indirect target of the effector Pi02860 through its association with the S factor StNRL1, which is a predicted ubiquitin E3 ligase. We thus focused on SWAP70 in this study as a potential substrate of StNRL1. To confirm this interaction in yeast, a full-length StSWAP70 prey construct was tested pairwise with bait constructs for StNRL1 and the empty bait vector. While all transformants grew on the control plates, only yeast containing both StSWAP70 and StNRL1 were able to grow on the selection plates and activate the β-galactosidase (β-Gal) reporter (Fig. 1A).

In addition, the Y2H screen with StNRL1 also generated 14 yeast cotransformants containing prey constructs expressing StNRL1 itself (SI Appendix, Table S1). Yeast expressing StNRL1 in both bait and prey vectors were able to grow on selection plates and activate the β-Gal reporter (Fig. 1A), suggesting that StNRL1 forms a homodimer. This is consistent with the role of BTB/POZ domain-containing proteins, which form homodimers as a core component of a CUL3-based ubiquitin protein ligase (E3) enzyme complex (26, 27).

To confirm that these interactions also occur in planta, coimmunoprecipitation (co-IP) assays were performed by expressing either GFP-StSWAP70 or GFP-StNRL1 with cMYC-GUS, cMYC- EV, or cMYC-StNRL1, pulling down with GFP-TRAP_M beads. Whereas all proteins were present in the relevant input samples, only cMYC-StNRL1 was coimmunoprecipitated in the presence of GFP-StSWAP70 (Fig. 1B and SI Appendix, Fig. S1) or GFP-StNRL1 (Fig. 1C and SI Appendix, Fig. S1). Previously, we showed that cMYC-StNRL1 did not bind nonspecifically to GFP beads (22). Thus, StNRL1 specifically associates with itself and with StSWAP70 in planta.

StSWAP70 Localizes to Endosomes. GFP was fused to the N terminus of StSWAP70 (GFP-StSWAP70) and expressed in N. benthamiana. The fusion protein was observed to localize to vesicles, with cytoplasmic background (Fig. 1D). To further characterize the nature of the vesicles, a series of colocalization experiments were conducted with subcellular markers representing...
SI Appendix, Fig. S2). Two independent VIGS constructs, TRV: SWAP70 V1 and TRV:SWAP70 V2, were generated to specifically silence NsSWAP70 by cloning two portions of the gene in the possible DH domain, which is downstream of the PH domain (SI Appendix, Fig. S3A). qRT-PCR was used to test silencing levels in each of three independent biological replicates and showed that transcript levels of NsSWAP70 were reduced by 50–80% in plants expressing each TRV:SWAP70 construct compared with plants expressing the TRV:GFP control (SI Appendix, Fig. S3B).

Because both Pi02860 and StNRL1 were observed to suppress ICD when transiently expressed in N. benthamiana (22), this prompted us to investigate whether StSWAP70 is required for ICD. Constructs expressing INF1 were agroinfiltrated into plants expressing each of the VIGS constructs and cell death was scored at 6 d postinoculation (dpi). At 6 dpi, a significant decrease (ANOVA, P < 0.001) developing compared with the TRV:GFP control plants (Fig. 2C). Moreover, significantly more P. infestans sporangia (ANOVA, P < 0.001) were produced (Fig. 2B). This indicates that StSWAP70 is detrimental to P. infestans infection.

We investigated whether StSWAP70 overexpression could alter levels of P. infestans colonization or ICD. Transient expression in N. benthamiana of GFP-StSWAP70, followed by pathogen challenge, was found to result in significantly smaller P. infestans lesions (ANOVA, P < 0.001) compared with free GFP expression (Fig. 2D), which is consistent with it acting as a positive regulator of immunity. Moreover, GFP-StSWAP70 coexpression with INF1 significantly (ANOVA, P < 0.001) accelerated ICD compared with free GFP (Fig. 2E), whereas GFP-StNRL1 expression had a significantly suppressive effect on ICD (ANOVA, P < 0.001), as shown recently (22).

Previously, we demonstrated that Pi02860 targets the S factor StNRL1 to suppress ICD (22). As anticipated, there were no significant differences in Cf4-Avr4 cell death in TRV:SWAP70 V1 or TRV:SWAP70 V2 plants compared with TRV:GFP control plants (Fig. 2C). The same assay was carried out to examine the cell death triggered by coexpression of the potato C4 resistance gene with Cladosporium fulvum Avr4, which was shown not to be suppressed by either Pi02860 or StNRL1 (22). As anticipated, there were no significant differences in Cf4-Avr4 cell death in TRV:SWAP70 V1 or TRV:SWAP70 V2, compared with the TRV:GFP control plants (Fig. 2C). These results confirm observations in the literature that SWAP70 is a positive regulator of immunity (24, 25) and reveal that it is required for ICD.
StNRL1 is an endogenous negative regulator of StSWAP70 that is exploited by the effector Pi02860.

**PI02860 and StNRL1 Promote StSWAP70 Turnover.** To ascertain the biological significance in defense suppression of the interaction between putative ubiquitin E3 ligase StNRL1 and StSWAP70, we investigated the protein levels of StSWAP70 in *N. benthamiana* when coexpressed with StNRL1 in the presence or absence of Pi02860, and also with or without treatment with the 26S proteasome inhibitor MG132. Immunoblot analysis revealed that the abundance of GFP-StSWAP70 was reproducibly decreased when coexpressed with cMYC-Pi02860 or cMYC-StNRL1, but this effect was prevented by MG132. No such reduction in StSWAP70 level was seen when GFP-StSWAP70 was coexpressed with cMYC-GUS, or coexpressed with cMYC-empty vector (EV) (Fig. 3A and SI Appendix, Fig. S4). Interestingly, the protein level of GFP-StSWAP70 was consistently barely detectable when coexpressed with StNRL1 and Pi02860 together, and this degradation was again attenuated by MG132 treatment (Fig. 3A and SI Appendix, Fig. S4). These observations suggest that the GEF, StSWAP70, is destabilized by Pi02860 and StNRL1 through proteasome-dependent degradation.

**PI02860-Mediated Degradation of StSWAP70 Is NRL1-Dependent.** NRL1 is a susceptibility factor, the activity of which is required for Pi02860 to promote *P. infestans* colonization and suppress ICD (22). To determine whether Pi02860-mediated degradation of StSWAP70 is NRL1-dependent, we thus investigated StSWAP70 protein level on NRL1 silenced *N. benthamiana*. We coinfiltred Agrobacteria expressing GFP-StSWAP70 with either cMYC-EV or cMYC-Pi02860 into plants expressing TRV (control), or VIGS constructs TRV:NRL1 V1 or TRV:NRL1 V2 (22) to silence NbNRL1. Immunoblot analysis showed that GFP-StSWAP70 is stable when coexpressed with cMYC-EV either on the TRV:GFP plants or NbNRL1-silenced plants (Fig. 3B and SI Appendix, Fig. S5). As anticipated, GFP-StSWAP70 abundance was reduced by cMYC-Pi02860 but not cMYC-EV on the TRV:GFP control plants. In contrast, GFP-StSWAP70 abundance was reproducibly unaltered when coexpressed with cMYC-Pi02860 on the VIGS plants in which NbNRL1 was silenced (expressing TRV:NRL1 V1 and TRV:NRL1 V2) (Fig. 3B and SI Appendix, Fig. S5), demonstrating that silencing of NRL1 prevented Pi02860-stimulated turnover of StSWAP70. This indicates that proteasome-mediated degradation of StSWAP70 is NRL1-dependent.

**Proteasome-Mediated Degradation of StSWAP70 Requires Dimerization of StNRL1.** NRL1 belongs to a large protein family that includes the functionally characterized members NPH3 and RTP2 in *Arabidopsis*, which are required for ubiquitination, internalization, and proteasome-mediated turnover of phototropins to regulate blue-light signaling (32). The BTB/POZ domain in NPH3 promotes an association with CULLIN3 (CUL3), forming a substrate adaptor in a CRL3 complex (CRL3*NPH3*) that targets phototropin 1 (phot1) for ubiquitination (32, 33). As a negative regulator of immunity, NRL1 is thus predicted to act as a substrate adaptor in a CRL3*NRL1* complex that targets proteins associated with immunity for degradation (22).

Dimerization of BTB proteins is required for selection of substrates for ubiquitination. Thus, mutations in conserved pocket residues in the BTB domain that prevent or reduce dimerization result in loss of substrate interaction and ubiquitination (34, 35). Indeed, we have recently shown that mutation to prevent dimerization of the BTB/POZ domain in E3 ligase POB1 prevents interaction with, and proteasome-mediated turnover of, its substrate PUB17 (36). We identified the conserved residues in StNRL1 (Asp28 and Lys42) (*SI Appendix, Fig. S6A*) and mutated Asp28 (D28) to Asn (N) and Lys42 (K42) to Gln (Q), generating StNRL1*NO* mutant. The effect of this mutation on StNRL1 dimerization was tested using co-IP. When the fusion constructs GFP-StNRL1 and cMYC-StNRL1 were coexpressed, the latter was strongly coimmunoprecipitated. In contrast, GFP-StNRL1 or GFP-StNRL1*NO* showed considerably weakened associations with cMYC-StNRL1*NO* (*SI Appendix, Fig. S6B*). This confirms the effect of the D28N/K42Q mutation in attenuating NRL1-mediated dimerization in planta. As anticipated, whereas GFP-StSWAP70 associated with cMYC-StNRL1 wild-type, which was accompanied by reduced GFP-StSWAP70 abundance, no such association was observed between GFP-StSWAP70 and cMYC-StNRL1*NO* mutant (Fig. 4A), suggesting that dimerization is required for StNRL1 to associate with StSWAP70. However, the interaction of the GFP-Pi02860 effector fusion was maintained in planta with the FLAG-StNRL1*NO* mutant (*SI Appendix, Fig. S6C*).

To examine the effect of StNRL1*NO* mutation on the ability of Pi02860 to mediate StSWAP70 degradation, FLAG-StNRL1*NO* and FLAG-StNRL1 were coexpressed in *N. benthamiana* with GFP-StSWAP70 and cMYC-Pi02860, with or without MG132 treatment (Fig. 4B and *SI Appendix, Fig. S7*). As expected, GFP-StSWAP70 protein abundance was reproducibly reduced by coexpression with cMYC-Pi02860 and FLAG-StNRL1, and this was inhibited by MG132 (Fig. 4B and *SI Appendix, Fig. S7*). In contrast, GFP-StSWAP70 protein abundance was unaffected by coexpression with FLAG-NRL1*NO* mutant and cMYC-Pi02860 (Fig. 4B and *SI Appendix, Fig. S7*). This indicates that StNRL1*NO* acts as a dominant-negative mutation; although its interaction is maintained with the effector Pi02860, its homodimerization is attenuated and thus StNRL1*NO* fails to interact with StSWAP70 to mediate its degradation.
To investigate whether the StNRL1<sup>NO</sup> mutant could suppress ICD, GFP-EV (control), GFP-StNRL1<sup>NO</sup>, and GFP-StNRL1<sup>NO</sup> were each transiently coexpressed with INF1 on <i>N. benthamiana</i> leaves. The ability of GFP-StNRL1<sup>NO</sup> to suppress ICD was significantly reduced (ANOVA, <i>P < 0.001</i>) compared with the GFP-StNRL1 wild-type (<i>SI Appendix</i>, Fig. S8A). Previously, we have shown that silencing of NRL1 in <i>N. benthamiana</i> prevented effector Pii02860 from enhancing colonization (22). We thus investigated the impact of the NRL1<sup>NO</sup> mutant on late blight disease. Critically, using a high inoculum of <i>P. infestans</i> zoospores, whereas transient expression of GFP-StNRL1 led to enhanced <i>P. infestans</i> colonization, expression of GFP-StNRL1<sup>NO</sup> mutant attenuated <i>P. infestans</i> leaf colonization compared with the GFP control (Fig. 4C). These results indicate that both the dimerization of StNRL1 and its ability to interact with StSWAP70 are essential for its function in suppressing the ICD immune response and thus in enhancing <i>P. infestans</i> infection.

To examine whether the StNRL1<sup>NO</sup> mutant had an effect on the ability of Pii02860 to suppress ICD, INF1 was coexpressed in <i>N. benthamiana</i> leaves with GFP-EV control, GFP-Pii02860 alone, GFP-Pii02860 with wild-type GFP-StNRL1, or GFP-Pii02860 with GFP-StNRL1<sup>NO</sup>. At 6 dpi, GFP-Pii02860 alone suppressed ICD to a significant level (<i>P < 0.001</i>) compared with free GFP (<i>SI Appendix</i>, Fig. S8B), as anticipated. However, the suppression of ICD was significantly enhanced (ANOVA, <i>P < 0.05</i>) by coexpression of GFP-Pii02860 with wild-type GFP-NRL1, whereas the suppression of ICD was significantly reduced (ANOVA, <i>P < 0.05</i>) when GFP-Pii02860 was coexpressed with GFP-StNRL1<sup>NO</sup> (<i>SI Appendix</i>, Fig. S8C). This confirms that the dimerization-deficient NRL1<sup>NO</sup> acts as a dominant-negative to reduce suppression of ICD by Pii02860.

**A Pii02860 Mutant That Fails to Interact with StNRL1 Does Not Promote StSWAP70 Degradation.** To provide additional genetic evidence linking Pii02860 activity to StNRL1 interaction and StSWAP70 turnover, we attempted to make an effector mutant that was unable to interact with StNRL1. Because the C terminus of RXXR effectors is regarded as the effector domain, we made sequential 5- and 10-amino acid deletions from the C terminus (<i>SI Appendix</i>, Fig. S9A) and tested whether interaction with StNRL1 was maintained using the Y2H assay. We found that, whereas a 5-amino acid deletion (Pii02860<sub>Δ126–131</sub>) retained weak interaction indicated by activation of the β-Gal assay, a 10-amino acid deletion (Pii02860<sub>Δ126–135</sub>) no longer interacted (<i>SI Appendix</i>, Fig. S9B). We confirmed that the mutant Pii02860<sub>Δ126–135</sub> form also failed to interact with StNRL1 in planta, although it was as stable as the wild-type Pii02860 form (Fig. 5A). Unlike the wild-type effector, Pii02860<sub>Δ126–135</sub> also failed to enhance <i>P. infestans</i> colonization of <i>N. benthamiana</i> (Fig. 5B), suppress ICD (Fig. 5C), or promote turnover of StSWAP70 (Fig. 5D).

**Pii02860 Enhances the Association Between StNRL1 and StSWAP70.** To examine the effect of Pii02860 on the interaction between StNRL1 and StSWAP70, we conducted a co-IP assay of GFP-SSiSWAP70 and cMYC-StNRL1 in the presence or absence of cMYC-Pii02860. GFP-SSiSWAP70 was coexpressed with cMYC-EV, with cMYC-GUS, with cMYC-StNRL1 alone, or with cMYC-StNRL1 and cMYC-Pii02860 together and pulled down using GFP-TRAP_M beads. As observed previously in Fig. 3,
while all proteins were present in the relevant input fractions, the protein level of GFP-StSWAP70 was decreased by coexpression with cMYC-StNRL1, and was further reduced when coexpressed with both cMYC-StNRL1 and cMYC-Pi02860 (Fig. 6 and SI Appendix, Fig. S10). As observed in Fig. 1C, cMYC-StNRL1 was coimmunoprecipitated with GFP-StSWAP70. Critically, however, reproducibly more cMYC-StNRL1 protein was immuno-precipitated by GFP-StSWAP70 in the presence of Pi02860 even though there is a corresponding decrease in the abundance of GFP-StSWAP70 in the input samples (Fig. 6 and SI Appendix, Fig. S10). However, the mutated effector Pi02860Δ126–135 was unable to enhance the association of StNRL1 with StSWAP70 (Fig. 6).

**Discussion**

We have recently shown that the RXLR effector Pi02860 promotes virulence by interacting with the CUL3 E3 ligase substrate adaptor protein StNRL1, exploiting its ability to negatively regulate immunity (22). Here we show that the Rho-GEF protein StSWAP70, a positive regulator of immunity, interacts with StNRL1 and is a substrate for proteasome-mediated degradation that is dependent on StNRL1.

The Y2H screen with StNRL1 revealed not only StSWAP70 as an interactor but also NRL1 itself, 14-3-3 proteins, PP2A, and a HAT4-like homeobox protein (SI Appendix, Table S1). Interestingly, 14-3-3 proteins (37, 38), and NRLs (37), have all been found to directly interact with blue-light phototropin receptors Phot1 and Phot2 in yeast and *in planta*. Interaction between PP2A and Phot2 has been implicated in blue-light-induced chloroplast movement (40). Critically, the *Arabidopsis* ortholog of StNRL1, NCH1, has recently been shown to be required for light-stimulated chloroplast accumulation (41). Future work will reveal whether StNRL1 plays a role in chloroplast movement responses to light, or indeed to pathogen challenge, and whether any of the interacting proteins in addition to StSWAP70 are potential substrates for proteasome-mediated degradation. However, StSWAP70 dominated the Y2H screen along with NRL1 itself, and became our focus here, as StSWAP70 has been reported to play a positive role in regulating immunity (24, 25).

In our work we show that the effector Pi02860 enhances the interaction between the CUL3 E3 ligase adaptor protein StNRL1 and StSWAP70, promoting a rapid turnover of StSWAP70, leading to susceptibility (Fig. 6B). The pathogen effector Avr-Piz-t from *Magnaporthe oryzae* also directly interacts with ubiquitin E3 ligases APIP6 and APIP10 in rice (42, 43). However, in contrast to NRL1, APIP6 and APIP10 are positive regulators of PTI and interaction with Avr-Piz-t results in their degradation, and consequent PTI suppression. Interestingly, the resistance protein Piz-t is a substrate for ubiquitination and degradation by...
AP1P10. Therefore, AP1P10 degradation promoted by Avr-Piz-t results in increased stability of Piz-t, leading to ETI (43).

The phytoplasma effector Sap54 also mediates degradation of a host protein. Sap54 interacts with MADS-domain transcription factors (MTFs), which regulate floral development, and also interacts with members of the RADIATION SENSITIVE23 (RAD23) family, which shuttle substrates to the proteasome for degradation (44). As a consequence, the MTFs are degraded. No known association between RAD23 and MTFs exist, and RAD23 proteins are not known to regulate flowering and Maclean et al. (44) concluded that Sap54 provided a “short-circuit,” bringing together two plant proteins that do not normally associate. In contrast, StNRL1 interacts directly in Y2H with StSWAP70, an interaction that is maintained in planta. Moreover, in the absence of the effector Pi02860, StNRL1 is an endogenous negative regulator of immunity that promotes StSWAP70 turnover. The effector Pi02860 was observed to enhance the StNRL1–StSWAP70 interaction. It is possible that, during an immune response, negative regulators such as StNRL1 are simultaneously inactivated, or are prevented from targeting substrates that are required for immunity. Future work will investigate how StNRL1 is regulated and whether Pi02860 interferes with that, or whether the observed strengthening of StNRL1–StSWAP70 interaction is its primary mode of action.

We show that StSWAP70 is a positive regulator of immunity, in the form of ICD, and that silencing it leads to enhanced P. infestans infection. Previously it has been shown in rice that OsSWAP70 interacts with Rho GTase OsRac1 and its DH domain activates OsRac1 by promoting dissociation of GDP to facilitate the association of GTP (24). The interconversion of GDP-bound inactive and GTP-bound active forms of Rac1 acts as a molecular switch in the regulation of many cellular processes, including PTI and ETI (45). Rac1 can be activated by different GEFs, including SWAP70, that are themselves activated via phosphorylation by distinct immune receptors (45, 46). In rice, for example, OsRacGEF1 has been shown to act downstream of the chitin receptor complex OsCEBiP/OsCERCK1 to activate OsRac1 (46). Here we show that StSWAP70 is required for ICD but not for cell death triggered by perception of C. fulvum Avr4 by C4. It is thus possible that StSWAP70 is activated via phosphorylation by receptors that perceive elicitors such as INF1.

As a GEF, StSWAP70 is implicated in activating small GTPases, such as Rac1, but precisely how it regulates immunity is poorly understood. It is important to note that the interaction between NRL1 and Pi02860 was shown to occur primarily at the host plasma membrane (22). This is consistent with other NRLs, such as NPF3, which interact with ARF-GEF proteins to regulate endocytosis of the auxin PIN receptors (32). It was thus perhaps unsurprising to find that StSWAP70 localized to endosomes (Fig. 1), and future work should investigate the impact of Pi02860 and StNRL1 upon the endocytosis of the elicitor receptor ELR. Receptor internalization is required for immune signaling (47). The endocytic cycle has been shown to be a target for pathogens. For example, the type III effector HopM1 from Pseudomonas syringae promotes the proteasome-dependent degradation of AtMIN7, a host ADP ribosylation factor and GEF that is required to regulate vesicle trafficking during PTI and ETI (48, 49). The exact mode of action of HopM1 remains unknown, but it will be interesting to see whether an E3 ligase such as NRL1 is coopted for its activity in removing AtMIN7.

Suppression of ICD has been observed in the presence of several P. infestans RXLR effectors. AVR3a suppresses it by targeting the ubiquitin E3 ligase CMPG1 and preventing its normal activity (23). More recently, we have shown that AVR2 activates a transcriptional regulator, CHL1, which is an endogenous negative regulator of ICD (13), and Pi17316 interacts directly with the potato VASCULAR HIGHWAY 1 (VH1)-interacting kinase (StVIK) as an S factor to suppress ICD (50). Now we show that the effector Pi02860 suppresses ICD by targeting the GEF protein SWAP70 for degradation by NRL1, an E3 ligase related to the blue-light regulators NPF3 and RPT2. This emphasizes the importance of the ICD immune pathway to late blight resistance, and highlights the diverse range of mechanisms employed to suppress it.
Recently, *P. infestans* effectors have been shown to target and exploit S factors to negatively regulate immunity (reviewed in ref. 5). Effector PI04314 forms unique holoenzymes with PP1c, presumably to dephosphorylate host proteins to suppress immunity (12). To date, the targets of PI04314-PP1c holoenzymes have not been identified. PI04089 effector targets the K-homology RNA binding protein KRBP1, increasing its stability and potentially promoting its activity (11). How the precise RNAs that interact with KRBP1 have yet to be identified. Here, we detail the mechanism underlying the activity of PI02860, which interacts with the ubiquitin E3 ligase NRL1, and reveal that SWAP70 is then targeted for removal to suppress immunity (Fig. 6B). Critically, preventing the degradation of SWAP70 either by silencing NRL1 or by expression of the demization-deficient mutant NRL1<sup>NQ</sup>, which interacts with the effector PI02860 but not the substrate SWAP70, provides a strategy to combat potato late blight.

Materials and Methods

Plant Materials and Growth Conditions. *N. benthamiana* plants were grown under a 16-h day at 22 °C and an 8-h night at 18 °C; when the ambient light dropped below 200 Wm<sup>-2</sup>, supplementary lighting was automatically provided, and above 450 Wm<sup>-2</sup> shading was automatically provided. Three-week-old plantlets were transplanted and grown in individual pots in a greenhouse at 20–26 °C, as described in ref. 23.

Plasmid Constructs. Full-length SISWAP70 was cloned from potato cDNA with gene-specific primers modified to contain the Gateway (Invitrogen) attB recombination sites. PCR products were purified and recombined into pDONR201 (Invitrogen) to generate entry clones via BP reactions using Gateway technology (Invitrogen). Primer sequences are shown in SI Appendix, Table S2. Protein fusions were made by recombinating the entry clones with the following plant expression vectors using LR clonase (Invitrogen): N-terminal GFP, mRFP, cMYC, FLAG fusions were made by recombinating the entry clones with pB7WG2F, pK7WG2R and pGW818, pGW412 respectively.

Mutagenesis. The STNRL<sup>NQ</sup> mutant was generated according to the manufacturer's protocol QuickChange Site-Directed Mutagenesis Kit (Stratagen) using pDONR201-STNRL1 as a template. The primers sequences used for mutation are shown in SI Appendix, Table S2. Two conserved amino acids of STNRL1, Asp at 28 position (D28) to Asn (N) and Lys at 42 position (K42) to Gin (Q), generating STNRL1<sup>NQ</sup> mutant, resulting in the mutant STNRL<sup>NQ,K42Q</sup>. The mutant STNRL1 was recombinated, using LR clonase, into pB7WG2F, PGWB412, or pGW818 for in planta assays.

**P. infestans** Infection Assay. *P. infestans* strains grown for 13 d are used for pathology tests. The plates were flooded with 10 mM MES, 10 mM MgCl<sub>2</sub> and 200 mM acetic acid, and adjusted to the required OD<sub>600</sub> before infiltration into *N. benthamiana* leaves [the OD<sub>600</sub> was generally 0.005–0.01 for confocal-imaging purposes, and 0.5 for immunoblots, IP, and homologous recombination (HR) activity assays]. For coexpression of multiple constructs, agroinfiltration carrying the different constructs were thoroughly mixed before infiltration. For agroinfiltration and infection assays, *Agrobacterium* suspensions at concentrations of OD<sub>600</sub> = 0.5 were infiltrated into 80,000 sporangia per milliliter after 1-d infiltration. Lesion sizes were measured at 7 dpi. The number of positive HRS (i.e., more than 50% of the inoculated sites forming clear cell death) were counted as described previously (56) and expressed as the mean percentage of total inoculations per plant. One-way ANOVA was performed to determine statistically significant differences.

Immunoprecipitation. N-terminal GFP-SISWAP70/cMYC-STNRL1 with or without cMYC-Pi02860, GFP-SNRL1/cMYC-STNRL1, mRFP-SNRL1/GFP-STNRL1, mRFP-SNRL1<sup>K42Q</sup>/FLAG-STNRL1, GFP-SISWAP70/cMYC-STNRL1<sup>K42Q</sup>, and all of the control combinations (Figs. 1C, 4B, and 5C, and SI Appendix, Figs. S3 and S7) were overexpressed in *N. benthamiana* using *Agrobacterium*-mediated expression. Leaf samples were collected at 48 h postinfiltration and proteins were extracted using G-TEN [10% (vol/vol) glycerol, 25 mM Tris·HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl] buffer with 10 mM DTT, protease inhibitor mixture, 1 mM phenylmethyl sulphonyl fluoride, and 0.2% Nonidet P-40. The fusions of GFP-tagged SISWAP70/cMYC-STNRL1 with or without cMYC-Pi02860, GFP-SISWAP70/cMYC-STNRL1<sup>K42Q</sup>, and GFP-SISWAP70/cMYC-STNRL1<sup>K42Q</sup> were immunoprecipitated using GFP-Trap-M magnetic beads (Chromotek). The mRFP-SNRL1/FLAG-SNRL1 or mRFP-SNRL1<sup>K42Q</sup>/FLAG-STNRL1<sup>NQ</sup> were immunoprecipitated using mRFP-Trap-M magnetic beads (Chromotek). The resulting samples were separated by PAGE and Western-blotted. Immunoprecipitated GFP fusion proteins, cMYC, FLAG, or mRFP fusions were detected using appropriate antisera (Santa Cruz Biotechnology). The ip-protoblot is described in Boevink et al. (12).
A Y2H screen was performed in Saccharomyces cerevisiae strain MaV203 as described in ref. 36 using the Invitrogen ProQuest system. DNA-binding domain “bait” fusions were generated by recombination between pDon201-StNRL1 and pDEST32, resulting in pDEST32-StNRL1. This construct was transformed into yeast strain MaV203, and nutritional selection used to recover transformants. A single transformant was grown up and used to prepare a cDNA library, which were transformed with a potato Y2H “prey” library, as described in ref. 12. Interactions were confirmed using reporter gene assays, namely the ability to grow on media missing histidine (−His), and screening for gain of β-Gal. The full-length coding sequence of the candidate interacting prey sequence, StSWAP70 (XM_006364745.1 (T398A, T917C); XP_006364807.1 (L133Q, L3065)), was cloned and stably transfected into pDEST32-StNRL1 and pDEST32-EV as a control to rule out the possibility that the observed reporter gene activation had resulted from interactions between the prey and the DNA-binding domain of the bait construct or the DNA-binding activity of the prey itself. StNRL1 was also cloned into the prey vector to obtain pDEST2-StNRL1, which was tested pairwise for an interaction with pDEST32-StNRL1 using pDEST2-EV as a control.

Confocal Imaging. A. tumefaciens containing GFP-StSWAP70 was co-infiltrated at a low OD600 (0.01–0.05) with different subcellular markers (ST- mRFP, mRFP-Ara7, Arab-mRFP, CFPS1) (57) into leaves of 4-week-old N. benthamiana plants. Cells expressing fluorescent protein fusions were observed using a Zeiss 710 confocal microscope with a PL APo/1.0 water dipping objective at no more than 2 d postinfiltration. GFP was excited by 488-nm light and its emissions were detected between 500 and 530 nm. mRFP was excited with 561 nm and its emissions were detected between 600 and 630 nm. CFPS was excited with 405 nm and its emission was detected between 440 and 480 nm.

Gene-Expression Assay. Total RNA was extracted from the leaves of N. benthamiana VIGS plants with an RNeasy plant kit (Qiagen) according to the manufacturer’s recommendations. Two milligrams of RNA was used for first-strand cDNA synthesizing using SuperScript II RNase HReverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time qRT-PCR was performed using Power SYBR Green and run on a Chromo4 thermal cycle (MJ Research) using OptionMonitor 3 software. Primer sequences are given in SI Appendix, Table S2. Primers were designed outside the region of cDNA targeted for silencing. Gene expression levels were calculated by a comparative Ct method, as described by ref. 58.

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