Natural Genetic Diversity in the Potato Resistance Gene *RB* Confers Suppression Avoidance from *Phytophthora* Effector IPI-O4

Hari S. Karki,¹ Sidrat Abdullah,² Yu Chen,² and Dennis A. Halterman^{1,†}

¹ United States Department of Agriculture–Agricultural Research Service, Madison, WI 53706, U.S.A.

² Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.

Accepted 6 May 2021.

RB is a potato gene that provides resistance to a broad spectrum of genotypes of the late blight pathogen *Phytophthora infestans*. *RB* belongs to the CC-NB-LRR (coiled-coil, nucleotide-binding, leucine-rich repeat) class of resistance (*R*) genes, a major component of the plant immune system. The *RB* protein detects the presence of class I and II IPI-O effectors from *P. infestans* to initiate a hypersensitive resistance response, but this activity is suppressed in the presence of the Class III effector IPI-O4. Using natural genetic variation of *RB* within potato wild relatives, we identified two amino acids in the CC domain that alter interactions needed for suppression of resistance by IPI-O4. We have found that separate modification of these amino acids in *RB* can diminish or expand the resistance capability of this protein against *P. infestans* in both *Nicotiana benthamiana* and potato. Our results demonstrate that increased knowledge of the molecular mechanisms that determine resistance activation and *R* protein suppression by effectors can be utilized to tailor-engineer genes with the potential to provide increased durability.

Most plant resistance (*R*) proteins have nucleotide-binding (NB) and leucine-rich repeat (LRR) motifs and can be divided into two subclasses based on their amino-terminal domain (Jones and Dangl 2006). One subclass encodes a coil-coil (CC) domain, while the other contains homology to the toll-interleukin-1 receptor (TIR) (Jones and Dangl 2006). The N-terminal domain (TIR/CC) of plant NB-LRR proteins has been proposed to initiate signaling or recognition (Eitas and Dangl 2010). Some host accessory proteins, which interact with both the *R* protein through the

N-terminal domain and the cognate pathogen effectors, have been demonstrated to be required for disease resistance (Eitas and Dangl 2010). To date, many effectors have been identified that can be recognized by the cognate host *R* proteins and activate effector-triggered immunity (ETI) upon recognition (Jones and Dangl 2006). Foundational progress has also been made in elucidating the mechanisms of *R* protein-effector interaction. The “guard,” “decoy,” and “bait-and-switch” models have been proposed to explain the recognition mechanisms, and each of them is supported in multiple pathosystems (Collier and Moffett 2009; Paulus and van der Hoon 2018; van der Hoon and Kamoun 2008).

Late blight of potato, caused by the oomycete pathogen *Phytophthora infestans*, continues to be one of the most challenging diseases to sustainably and proactively manage. The most frequently used management strategy to control late blight is to spray fungicides repeatedly, which is expensive and causes environmental concerns. Therefore, the best way to control the disease is through natural host resistance. Wild potato species have provided a rich source of resistance against late blight. *Solanum demissum* is a hexaploid Mexican species from which 11 *R* genes have been identified or cloned and extensively used in potato breeding for late blight resistance. However, virulent races of *P. infestans* have rapidly overcome most of the *R* genes from *S. demissum* in most potato-growing regions (Wastie 1991). Three major late blight resistance genes have also been cloned from another diploid wild potato species, *Solanum bulbocastanum*, including *RB* (Song et al. 2003), also known as *Rpi-blb1* (van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* (Lokossou et al. 2009).

The *P. infestans* effector *Avrblb1*, also known as IPI-O (in planta-induced gene *O*), was originally found from a screen for differentially expressed genes in *P. infestans* during potato infection (Pieterse et al. 1994; van West et al. 1998). IPI-O showed a high level of expression in invading hyphae (van West et al. 1998). Later studies revealed that IPI-O is a multigene effector family and the IPI-O locus can be extremely variable between isolates (Champouret et al. 2009; Halterman et al. 2010; van West et al. 1998). IPI-O variants have been divided into three classes based on diversity of their deduced amino acid sequences (Champouret et al. 2009; Halterman et al. 2010). In addition, *P. infestans* isolates lacking class I and class II variants are virulent on plants with *RB* (Champouret et al. 2009), and the *P. infestans* isolates with class III variants are more aggressive on plants with *RB* (Halterman et al. 2010). Interestingly, the class III variant IPI-O4 not only eludes detection by *RB* but is also capable of inhibiting the hypersensitive response (HR) elicited by class I variant IPI-O1 (Chen et al. 2012).

†Corresponding author: D. A. Halterman; dennis.halterman@usda.gov

Funding: This research was supported in part by the United States Department of Agriculture (USDA) Agricultural Research Service (ARS). Funding was provided through USDA National Institute of Food and Agriculture (NIFA) Plant-Associated Microbes and Plant-Microbe Interactions award number 2014-67013-21593, USDA-NIFA/National Science Foundation Plant Biotic Interactions Program award number 2018-67014-28488, and the ARS-State Cooperative Potato Research Program. The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or United States Government determination or policy.

*The e-Xtra logo stands for “electronic extra” and indicates there are supplementary materials published online.

The author(s) declare no conflict of interest.

Copyright © 2021 The Author(s). This is an open access article distributed under the CC BY-NC-ND 4.0 International license.

Despite the fact that *P. infestans* genotypes that overcome *RB* resistance have been found (Champouret et al. 2009; Halterman et al. 2010; Karki et al. 2021), *RB* remains valuable for potato breeding due to the almost ubiquitous existence of IPI-O1 in strains from major potato-growing regions. We have previously shown that *RB*-containing plants respond through elicitation of an HR after inoculation with *P. infestans* but differ in the transcription patterns for the HR-related gene *Hin1* and the pathogenesis-related (*PR*) genes *PR-1b*, *PR-2a*, and *PR-5* (Chen and Halterman 2011). These observations explain to some extent the partial resistance phenotype that has been repeatedly observed in plants with the *RB* gene and also suggest that *P. infestans* might be able to suppress responses in partially resistant plants with *RB* (Chen and Halterman 2011). Together with our previous discovery that IPI-O4 is able to inhibit the HR elicited by IPI-O1 through directly binding the RB CC domain (Chen et al. 2012), the data indicate a complex and dynamic interface between potato *R* gene products and *P. infestans* effectors. To elucidate the molecular mechanisms underlying resistance suppression, we investigated the intra- and intermolecular interactions between the RB protein and IPI-O effectors and proposed a model based on our observations (Chen et al. 2012). The RB protein forms a multimeric complex through intermolecular interactions of the CC domain, which is consistent with the “resistosome” model proposed by Wang et al. (2019a and b). In the absence of IPI-O1, RB remains in a resting state. Upon recognition of IPI-O1, a conformational change occurs, which enables RB to activate resistance, possibly by exposing a platform for signaling components. However, when IPI-O4 is present, this effector interacts with the CC domain and disrupts CC oligomerization or blocks an interaction with other signaling components, thus suppressing RB activation. This model illustrates that modification of the CC domain might be a useful strategy to engineer *RB* so that it can recognize more variants of IPI-O effectors or alter intermolecular CC domain interactions to prevent disruption by IPI-O4.

The current work is focused on identifying variation within the RB CC domain that avoids interaction with IPI-O4 and thus could enable resistance activation even in the presence of this suppressor. We found that modification of single amino acids within the RB CC domain can either diminish the resistance capability of the modified RB or increase the ability of RB to protect plants against *P. infestans* lineages that have been shown to overcome the wild-type RB protein. Our results demonstrate that increased knowledge of the molecular mechanisms that determine resistance activation and R protein suppression by effectors can be utilized to tailor-engineer genes with the potential to provide increased durability.

RESULTS

RB CC domain amino acids 115 and 117 influence multimerization in yeast.

We previously used natural variation of RB from wild species of potato to identify an RB CC domain from *S. pinnatisectum*

(pnt) that does not interact directly with IPI-O1 or IPI-O4 in a yeast two-hybrid assay (Chen et al. 2012). Amino acid sequence alignment of the CC domains of wild type RB and the RB ortholog from *S. pinnatisectum* (pnt) identified 21 amino acids that were different between the two (Fig. 1). Nine of these amino acids were nonconservative changes and were chosen for modification to test for their effect on intermolecular interactions. We performed site-directed mutagenesis to change the amino acids in the wild-type RB CC domain to the corresponding amino acids in the pnt RB. The modified RB CC domains were then tested for their ability to interact with the wild-type RB CC protein, IPI-O4, or self-interaction using a yeast two-hybrid assay (Supplementary Table S1). We found that single amino acid changes K115T and K117D diminished the ability of the CC domain to interact with IPI-O4 (Table 1). These mutations also affected self-association of the CC domains and their ability to interact with the wild-type RB CC domain. Mutation K131N also diminished interaction between the modified and wild-type RB CC domains. The double mutant K115T/K117D also was not able to interact with IPI-O4 in yeast.

RB CC domain amino acids 115 and 117 influence resistance activation.

We tested whether changes in the protein at positions 115 or 117, or both, within the RB CC domain still allow the protein to activate an HR elicited by IPI-O1. We created site-directed mutants at positions 115 and 117 of the wild-type RB gene under the control of a native promoter and terminator. *Nicotiana benthamiana* leaves were co-agroinfiltrated with either *RB115*, *RB117*, or *RB115/RB117* and IPI-O1. The *RB117* mutant retained HR, elucidating capability similar to the wild-type RB (Fig. 2A). However, *RB115* completely lost its ability to elicit HR upon recognition of IPI-O1. Coinfiltration of the *RB115/RB117* double mutant with IPI-O1 also elicited an HR, but initiation of HR was delayed compared with wild-type *RB* and the *RB117* mutant. None of the *RB* genes induced an HR when coinfiltrated with IPI-O4 alone (Fig. 2B), which is consistent with prior results

Table 1. Results of a yeast two-hybrid assay to test interactions of RB coiled-coil (CC) domain mutants with wild-type RB CC domain, itself, or IPI-O4

Amino acid mutation	RBCCblb	Self	IPI-O4
D29N	+	+	+
A43S	+	+	+
N55D	+	+	+
R85L	+	+	+
E90A	+	+	+
K115T	±	±	±
K117D	–	–	–
K131N	±	+	+
D155G	+	+	+
K158E	+	+	+
K115T, K117D	–	–	–
RBblb-CC wild type	+	+	+

Fig. 1. Amino acid alignment of the RB coiled-coil (CC) domain with the CC domain of the RB homolog from *Solanum pinnatisectum* (pnt). Amino acids highlighted in black, gray, and white indicate identical, similar, and nonconserved amino acids, respectively. Asterisks denote amino acids that were targeted for site-directed mutagenesis.

and shows that there is no gain of IPI-O4 recognition conferred by the K115T or K117D mutations. Previously, we reported that IPI-O4 suppresses the HR upon the recognition of IPI-O1 by RB (Chen et al. 2012). To test whether RB117 escapes suppression by IPI-O4, we coinfiltrated *N. benthamiana* leaves with either RB, RB115, RB117, or RB115/RB117 and IPI-O4, followed by infiltration of IPI-O1 at the same spot 24 h later. Our results show that the RB117 mutant retained its ability to elicit the HR upon recognition of IPI-O1 even in the presence of IPI-O4 (Fig. 2C). Despite the ability of the double mutant to elicit an HR upon recognition of IPI-O1, we found that coinfiltration with IPI-O4 led to suppression of the HR, suggesting that the 115 and 117 amino acids introduce subtle structural changes that modify the resistance protein's interactions with these effectors.

Introduction of the RB117 mutation does not affect resistance to *P. infestans* in *N. benthamiana*.

To determine whether mutations at locations 115 and 117 in the RB protein affect resistance against *P. infestans*, we agroinfiltrated the altered genes into leaves of 4- to 5-week-old *N. benthamiana* followed by inoculation with *P. infestans* US-23. The modified RB117 and RB115/RB117 genes conferred resistance to *P. infestans*, compared with RB115, which was susceptible (Fig. 3). The RB117 gene provided slightly improved resistance compared with the wild-type RB, but this difference was not substantial. These constructs were also co-agroinfiltrated with the IPI-O4 effector and were tested for their ability to confer resistance to *P. infestans* US-23. Our results were consistent with our prior results showing that, while IPI-O4 is capable of suppressing the elicitation of the HR, it is not able to block resistance to *P. infestans* completely (Chen and Halterman 2017a). Regions that were co-agroinfiltrated with wild-type RB, RB117, or RB115/117 along with IPI-O4 were resistant, while co-agroinfiltration of RB115 with IPI-O4 remained susceptible (Fig. 3).

RB117 provides resistance in potato.

Our results showing that RB with the K117D mutation confers resistance in *N. benthamiana* led us to test whether this gene is also functional against late blight in a potato pathosystem. We transformed potato cultivar Katahdin with either RB115 or RB117 under the control of an RB-native promoter and terminator. Although RB115 was not able to confer resistance in the transient expression assay, we transferred it into potato to serve as a control

in these experiments. We obtained 25 plantlets transformed with RB115 and 27 plantlets transformed with RB117 that successfully rooted on kanamycin-selective media. The plants were grown in the greenhouse and were observed for true-to-type growth habits compared with nontransformed Katahdin. Of the 25 RB115 transformants, ten survived in the greenhouse and exhibited a normal phenotype. Of the 27 RB117 transformants, 25 had a true-to-type phenotype. Fully developed, young, and healthy leaves from these plants were excised and used in a detached leaf assay (DLA) with *P. infestans* isolate US-23. After 5 days, the inoculated leaves were scored for late blight symptoms (Fig. 4). The majority of the RB115 plants were not significantly different from the susceptible wild-type Katahdin control. Three plants RB115(18), RB115(19), and RB115(20) exhibited a resistance phenotype similar to the SP951 resistant control. However, repeat experiments using older leaves from RB115(20) showed that it was susceptible to *P. infestans* (Supplementary Fig. S1), which is consistent with the previous assay using *N. benthamiana*. The majority of RB117 plants exhibited resistance, although RB117(29), RB117(32), and RB117(39) showed the fewest symptoms. Repeat experiments using older leaves of RB117(39) showed that it maintained resistance in older leaves (Supplementary Fig. S1).

RB117 maintains resistance in potato despite the presence of IPI-O4.

We previously showed that RB117 maintains its ability to elicit the HR and confer resistance to *P. infestans* in the presence of the IPI-O4 suppressor during transient coexpression in *N. benthamiana*. To test the effectiveness of RB117 in the presence of IPI-O4 in potato, we crossed the RB115(20) and RB117(39) plants with potato that is expressing the IPI-O4 effector under the control of a 35S promoter (Chen and Halterman 2017b). Progeny that were PCR-positive for both IPI-O4 and RB were used in a DLA to test for resistance to *P. infestans* US-23. Five progenies from the cross using RB115(20) and four progenies from the cross using RB117(39) were determined to contain IPI-O4. However, only one plant from each cross was found to have both IPI-O4 and RB. These plants were used in a DLA to determine the resistance phenotype using *P. infestans* US-23 (Fig. 5; Supplementary Table S2). The control plants, including susceptible Katahdin and Katahdin expressing IPI-O4, were both susceptible to *P. infestans*, while SP951 expressing the wild-type RB gene was considered resistant. Infected leaf area of both RB115(20) and RB115(20) + IPI-O4

Fig. 2. Effect of RB115 and RB117 mutations to elicit the hypersensitive response upon recognition of *Phytophthora infestans* effectors IPI-O1 and IPI-O4. **A**, Wild-type RB (wt), RB115, RB117, and double mutant RB115/117 coexpressed with IPI-O1. **B**, RB (wild type), RB115, RB117, and double mutant RB115/117 coexpressed with IPI-O4. **C**, RB (wild type), RB115, RB117, and double mutant RB115/117 coexpressed with IPI-O4, followed by infiltration in the same spot with IPI-O1 after 24 h. The numbers below each label indicate the number of attempts that exhibited cell death out of the total number of replications. Photographs were taken 6 days after the first infiltration.

plants was not significantly different from the susceptible Katahdin control. However, RB115(20) did exhibit some resistance, as it was also not significantly different from the resistant SP951 control. Consistent with our previous results, RB117(39) was significantly more resistant than Katahdin and showed fewer disease symptoms than SP951, but this difference was not significant. The RB117(39) + IPI-O4 plant was also resistant and was not significantly different from the RB117(39) parent plant.

Fig. 3. *Phytophthora infestans* resistance assay following transient expression of RB and RB mutants. Wild-type *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* containing RB115, RB117, or RB wild type either alone or coinfiltrated with IPI-O4. At 24 h after infiltration, the leaves were subjected to a detached leaf assay using *P. infestans* US-23. The percentage of infected leaf area was calculated 6 days after inoculation, using ImageJ.

RB117 does not provide resistance against *P. infestans* isolate NL13316 in potato.

We hypothesized that the inability of IPI-O4 to suppress RB117 could lead to increased resistance to more aggressive *P. infestans* genotypes. To test this, we conducted a DLA in *N. benthamiana* and transgenic potato expressing RB117 against the highly aggressive *P. infestans* isolate NL13316. This genotype has been shown to overcome RB resistance in potato (Karki et al. 2021). We first examined the susceptibility of *N. benthamiana* plants that had been stably transformed with the wild-type RB gene. We found that, similar to RB in potato, transgenic *N. benthamiana* was resistant to US-23 but susceptible to NL13316 (Fig. 6A). The infected leaf area of RB-transgenic *N. benthamiana* following inoculation with US-23 was significantly lower than the wild

Fig. 4. Results of a detached leaf assay using primary transformants of RB115 or RB117 in potato cultivar Katahdin inoculated with *Phytophthora infestans* US-23. Numbers in parentheses denote the individual plantlet that was excised from transformed tissue. Disease rating data are represented as violin graphs. Dashed lines indicate the median and dotted lines denote the quartiles. The thickness of each plot represents the frequency distribution of the data.

type ($P < 0.0001$). We found no significant difference between infected leaf area of the RB-transgenic *N. benthamiana* compared with the wild type using NL13316 ($P = 0.0616$). We then carried out transient expression of wild-type RB, RB115, and RB117 in *N. benthamiana* with and without IPI-O4, followed by inoculation with NL13316. We were surprised to find that transient expression of wild-type RB conferred resistance to NL13316, even in the presence of IPI-O4 (Fig. 6B). However, both RB115 and RB115 + IPI-O4 were susceptible to NL13316, ruling out the possibility of agrobacterium influencing the outcome of the interactions. Areas infiltrated with RB117 or the double mutant, with or without IPI-O4, were all resistant to NL13316 at a level similar to the wild-type RB.

In potato, a DLA was conducted using Katahdin + RB117, Katahdin + RB115, SP951, K41 (expressing the wild-type RB gene derived from somatic fusion with *S. bulbocastanum*), and nontransformed Katahdin inoculated with US-23 and NL13316 side by side on the same leaves (Fig. 7, Supplementary Table S3). Following inoculation with *P. infestans* US-23, we found no significant difference between the resistant RB117 and K41 leaves ($P = 0.9165$). SP951 showed partial resistance, as the infected leaf area was significantly different from both susceptible Katahdin ($P < 0.0001$) and resistant K41 ($P < 0.0001$). We

observed that along with K41 and SP951, RB117 was also susceptible to NL13316, as none of the infected leaf areas were significantly different from susceptible Katahdin. These results indicate that, although RB117 is able to escape IPI-O4 suppression and provide adequate resistance to US-23, this gene does not provide resistance against *P. infestans* isolate NL13316 in potato.

DISCUSSION

To suppress pathogen-associated molecular pattern-triggered immunity (PTI), pathogens introduce effector proteins into and around host cells (de Jonge et al. 2011; Giraldo and Valent 2013; Stergiopoulos and deWit 2009). Cytoplasmic pathogen effectors are recognized by intracellular NB LRR (NLR) receptors that trigger ETI (Dodds and Rathjen 2010; Jones and Dangl 2006). Effector recognition by R proteins is critical for the determination of resistance and susceptible phenotypes in plants by the pathogens. In this study, we have identified amino acids in the CC domain of the RB protein that are critical for molecular interactions that determine effector recognition and suppression by the *P. infestans* effector IPI-O4.

RB recognizes IPI-O variants belonging to class I (IPI-O1, IPI-O2, IPI-O5, IPI-O7, and IPI-O8) and class II (IPI-O3) (Champouret et al. 2009) to initiate a HR but does not recognize class III (IPI-O4) (Champouret et al. 2009; Halterman et al. 2010). Instead, IPI-O4 blocks RB-mediated recognition of IPI-O1 to induce cell death in *N. benthamiana* and IPI-O4 expressing *P. infestans* can overcome RB-mediated resistance in potato (Halterman et al. 2010). The tobacco N, *Arabidopsis* RPP1, and flax L5, L6, and L7 proteins all interact directly with their corresponding pathogen effector proteins (Dodds et al. 2006; Erickson et al. 1999; Krasileva et al. 2010). Similarly, we previously detected interaction between RB and the IPI-O1 effector in planta, using coimmunoprecipitation experiments, but this does not rule out the possibility that other host factors are involved in this interaction (Chen et al. 2012). The RB CC domain undergoes self-association and also physically interacts with IPI-O1 and IPI-O4 to turn resistance on and off (Chen et al. 2012). The role of the CC domain in the function of CC-NB-LRR proteins varies greatly. For example, the CC domain of RPM1 interacts with a host accessory protein RIN4 in *Arabidopsis*, which physically interacts with the cognate effectors *AvrRpm1* and *AvrB* (Mackey et al. 2002). In tomato, a CC-NB-LRR type of R protein Prf interacts with a host accessory protein Pto through the CC domain, and Pto interacts with the cognate effectors *AvrPto* or *AvrPtoB* physically (Mucyn et al. 2006). Overexpression of the CC domain of barley MLA alone can induce cell death in *N. benthamiana*, and this same domain also interacts with WRKY transcription factors to inhibit their repression of defense-related genes (Shen et al. 2007). Similarly, the CC domain of NRG1- and ADR1-like proteins of solanaceous species and *Arabidopsis thaliana* are sufficient to induce defense responses (Collier et al. 2011). In contrast, overexpression of the Rx CC domain alone does not induce an HR in *N. benthamiana*; however, along with the LRR domain, it coregulates the signaling activity of the NB domain (Rairdan et al. 2008).

The CC domain of RB is essential for HR elicitation in *N. benthamiana* and potato through its interaction with IPI-O1 (Chen et al. 2012). We have previously shown that IPI-O effectors such as IPI-O1 and IPI-O4 are able to interact with the vast majority of CC domains of RB from wild potatoes (Chen et al. 2012). The wild species accessions that harbored these RB CC domains do not contain functional full-length RB homologs and were only used as a source of natural genetic variation. Since interaction between IPI-O4 and the RB CC domain induces suppression of resistance, we used protein-protein interaction to screen for variation that could potentially escape IPI-O4 suppression. The CC domain of an RB homolog from *S. pinnatisectum* (pnt), RBpnt,

Fig. 5. Detached leaf assay of potato leaves with *Phytophthora infestans* isolate US-23. Detached leaves were inoculated with *P. infestans* isolate US-23 by dropping 10 μ l of a zoospore suspension on the abaxial side of the leaflets. Photographs were taken 5 days after inoculation and the percent diseased area of the leaflet was calculated using ImageJ.

does not interact directly with IPI-O1 and IPI-O4 in yeast (Chen et al. 2012). Sequence variation between the CC domains of RB and RBpnt identified 21 amino acid differences, nine of which conferred nonconservative changes. Using site-directed mutagenesis, we found that two of these amino acids at locations 115 and 117 affect CC domain dimerization and interaction with IPI-O4. Furthermore, we showed that amino acid 117 is likely a target for IPI-O4 suppression, as modification of the RB CC domain at this location allowed for resistance activation even in the presence of IPI-O4. Comprehensive mutational analysis in the *Arabidopsis* *RPS2* and *Pseudomonas syringae* *avr-Rpt2* system has shown that single amino acid substitution in *RPS2* can disrupt cell death mediated by the R protein-effector interaction (Axtell et al. 2001). Our result is in line with previous findings that targeted mutations in the R protein can expand the effector recognition spectrum

(Farnham and Baulcombe 2006; Harris et al. 2013; Segretin et al. 2014). Random mutagenesis of the potato NLR resistance protein R3a identified variation that allowed it to recognize the normally undetectable AVR3a^{EM} effector variant from *P. infestans*, while still maintaining recognition of the AVR3a^{KI} isoform (Segretin et al. 2014). This mutation, located within the NB pocket, resulted in a gain-of-function response in transient assays with the effectors in *N. benthamiana* but failed to improve resistance to *P. infestans* after transient or stable expression in *N. benthamiana* or potato (Segretin et al. 2014). Here, we found that stable expression of RB117 is able to provide resistance to *P. infestans* US-23 after stable expression in potato but is unable to withstand the RB-breaking genotype NL13316. Random mutagenesis of the potato Rx NLR protein identified variation that provided broader-spectrum resistance to potato virus X but led to an increased fitness

Fig. 6. Disease assay on nontransgenic (wild type [WT]) and RB transgenic *Nicotiana benthamiana*. **A**, Fully developed 4-week-old leaves were inoculated with the *Phytophthora infestans* isolates US-23 and NL13316. The percentage of infected leaf area was calculated 5 days after infection, using ImageJ. **B**, WT *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* containing either RB115, RB117, or RB WT with or without coinfiltration with IPI-O4. At 24 h after infiltration, the leaves were collected and were subjected to a detached leaf assay using *P. infestans* isolate NL13316. The percentage of infected leaf area was calculated 6 days after infection.

cost to the host (Farnham and Baulcombe 2006). Further mutational analysis identified variation within the R protein that could offset this fitness cost and led to a stronger resistance response (Harris et al. 2013). Despite these apparent limitations, modified R proteins that potentially expand their recognition spectrum are still useful in crop improvement. The combination of wild-type and modified R proteins could lead to an optimized defense against pathogens and make it more difficult for the pathogen to evolve to avoid recognition or suppress resistance.

The *RB* gene provides broad-spectrum resistance to a range of *P. infestans* genotypes (Song et al. 2003; van der Vossen et al. 2003). However, there are multiple reports of *P. infestans* isolates that can overcome the *RB* gene (Champouret et al. 2009; Halterman et al. 2010; Karki et al. 2021). Strategies to combat resistance-breaking genotypes of *P. infestans* can include stacking of R genes that confer recognition of essential effectors or effectors expressed at different developmental stages of the pathogen. Additionally, we provide support for an approach that includes improved R proteins that can avoid effector suppression or broaden recognition specificity. Our results indicate that, although RB117 escapes IPI-O4 suppression and is effective at limiting infection of *P. infestans* US-23, it did not provide adequate resistance against the *RB*-breaking genotype NL13316 in potato. The US-23 genome contains the IPI-O4 effector (Chen and Halterman 2017b) even though the wild-type *RB* gene is still effective against this genotype. Additionally, even overexpression of IPI-O4 in potato does not completely disrupt resistance in plants that contain RB (Chen and Halterman 2017a). This suggests that other factors within the pathogen can modulate the effectiveness of resistance suppression and virulence. The NL13316 genotype is relatively uncharacterized, and we look forward to understanding more about its ability to overcome RB-mediated resistance. Interestingly, we found that NL13316 could not overcome transiently expressed wild-type RB or RB117 in *N. benthamiana*. The expression constructs for both these genes contain the native *RB* promoter, so overexpression of the genes alone does not explain this phenomenon. This leaves open the possibility that the presence of *Agrobacterium tumefaciens* is affecting the ability of *RB* and RB117 to elicit resistance, perhaps by priming ETI through

PTI activation (Ngou et al. 2021). This further suggests that multiple factors beyond the binary RB/IPI-O interaction are necessary to determine the outcome of the host-pathogen encounter.

Late blight remains the devastating disease in the potato production system. The cloning of *Rpi* genes offers the possibility of using them in potato improvement programs using biotechnology. However, the lifespan of R genes is determined by the ability of the pathogen to evolve to avoid recognition or suppress defense responses. Molecular and mutational characterization of *RB* and its corresponding effector presented in this study demonstrate that engineering new alleles could strengthen the arsenal of R genes available for resistance breeding. Current advancements in genome editing technology can target specific nucleotide sequences and could provide an avenue to modify R genes without the requirement of the transferring nonplant sequences. Further functional and biochemical understanding of RB117 with several *P. infestans* isolates will assist in the use of this gene in late blight management and increased resistance durability.

MATERIALS AND METHODS

Plant materials and growth conditions.

Potato plants were grown in the greenhouse in soil-less potting mix (BM1; Berger) under greenhouse conditions (23°C day and 15°C night temperatures with 14 h of light). Wild-type and *RB*-transgenic *N. benthamiana* plants were grown in pots in a growth chamber with 28°C day and 27°C night temperatures and 16 h of light. All the plants were watered and fertilized regularly as needed.

Yeast two-hybrid assay and site-directed mutagenesis.

The CytoTrap system (Stratagene) was used to detect protein-protein interactions. Constructs used in this paper to test interactions are the same as published previously (Chen et al. 2012). Site directed mutagenesis of RB CC domains for interaction testing was done using the wild-type RB CC domain in the pMyr vector as a template. A pair of oligonucleotide primers containing the desired nucleotide substitution, each complementary to the opposite strands of the same target sequence, were extended during temperature cycling, by using PfuTurbo DNA polymerase (Stratagene). A list of designed primers is shown in Supplementary Table S4. The PCR conditions for substitution mutants were: 2 min at 95°C, followed by 18 cycles of 30 to 50 s at 95°C, 50 s at 72 to 75°C, 13 min at 68°C, and 7 min at 68°C. After the temperature cycling, the PCR products were treated with *DpnI* for 2 h at 37°C to digest the methylated parental DNA template. The resulting products were transformed into GC5 chemical competent cells (GeneChoice). Mutations at the targeted sites were verified by sequencing.

Mutations at locations 115 and 117 in the full-length RB gene were generated by circular PCR (as outlined above), using the RGA2-PCR long-range PCR product that was first cloned into the *Bam*HI site of pBluescript KS. The resultant mutated plasmid constructs were verified by sequencing, before subcloning the modified RB gene into the *Bam*HI site of the pCLD04541 vector and introduction into *A. tumefaciens* GV3101.

Generation of transgenic potatoes.

The RB-modified versions RB115 and RB117 were transferred into potato cultivar Katahdin as described previously (Bhaskar et al. 2008). The transgenic plants were maintained in tissue culture media and were propagated by cuttings in the greenhouse. The introduction of these genes was confirmed using RB-specific primers, and PCR conditions were as follows: 1 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 68°C, and 7 min at 68°C. Genomic DNA was extracted from the young growing leaves of potato plants with the DNeasy plant maxi kit (Qiagen), according

Fig. 7. Detached leaf assay of potatoes with *Phytophthora infestans* US-23 and NL13316. Each leaf was inoculated with US-23 on the left and NL13316 (NL) on the right. Photographs were taken 5 days after inoculation and the percentage of diseased area of the leaflet was calculated using ImageJ. **A**, Representative photos of leaves from the detached leaf assay. **B**, Percentages of diseased leaf areas.

to the manufacturer instructions. To transfer the RB117 into IPI-O4 genetic background (Chen and Halterman 2017b), we crossed the selected RB117 and RB115 transgenic potatoes to the IPI-O4-containing transgenic potato. The resulting F1 progenies were tested for the presence of IPI-O4, using primers specific for the effector and for RB (Supplementary Table S4).

***P. infestans* isolates and late blight infection assay.**

P. infestans isolates US-23 and NL13316 were used for late blight infection in a DLA. US-23 and NL13316 were provided by A. Gevens (University of Wisconsin, Madison, WI, U.S.A.) and Simplot Plant Sciences, respectively. *P. infestans* growth conditions, zoospore production, and infection assays were performed as described previously (Karki et al. 2021). Infected area in the leaves were quantified using ImageJ software (Rueden et al. 2017).

Disease and HR assay in *N. benthamiana*.

A 5-ml culture of *A. tumefaciens* carrying the various constructs was grown overnight at 28°C in media supplemented with proper antibiotics. *Agrobacterium* cells were harvested and resuspended in MMA induction buffer (1 liter MMA = 1g of Murashige Skoog salts, 1.95 g of MES, 20 g of sucrose, 200 µM acetosyringone, pH 5.6). All suspensions were incubated at room temperature for 2 h prior to infiltration. For the disease (late blight infection) assay, *A. tumefaciens* that had been transformed with the *R* genes at an optical density at 600 nm (OD₆₀₀) = 0.3 were infiltrated in the *N. benthamiana* leaves. At 24 h after infiltration, a DLA was performed with *P. infestans* isolates US-23 and NL13316 with zoospore concentrations of 50,000 per milliliter. The leaves were photographed 6 days after inoculation, unless otherwise stated, and infected areas in the leaves were quantified using ImageJ (Rueden et al. 2017).

For the assay, *R* genes and effectors were equally combined to a final OD₆₀₀ of 0.3. For the HR suppression assay by IPI-O4, first, RB, RB115, and RB117 were coinfiltrated with IPI-O4, then, 24 h later, IPI-O1 was infiltrated on the same spot as described by Chen et al. (2012). The infiltrated leaves were assessed for cell death and were photographed after 6 days. Two leaves from three to four plants were tested for each treatment and the experiment was replicated three times.

Statistical analyses.

Mean comparisons were performed using Kruskal-Wallis and a Dunn's multiple comparisons test. Grouped data (Figs. 6A and 7) were analyzed using a two-way analysis of variance and Šidák's multiple comparisons test.

LITERATURE CITED

- Axtell, M. J., McNellis, T. W., Mudgett, M. B., Hsu, C. S., and Staskawicz, B. J. 2001. Mutational analysis of the *Arabidopsis* *RPS2* disease resistance gene and the corresponding *Pseudomonas syringae* *avrRpt2* avirulence gene. *Mol. Plant-Microbe Interact.* 14:181-188.
- Bhaskar, P. B., Raasch, J. A., Kramer, L. C., Neumann, P., Wielgus, S. M., Austin-Phillips, S., and Jiang, J. 2008. *Sgt1*, but not *Rar1*, is essential for the RB-mediated broad-spectrum resistance to potato late blight. *BMC Plant Biol.* 8:8.
- Champouret, N., Bouwmeester, K., Rietman, H., van der Lee, T., Maliepaard, C., Heupink, A., van de Vondervoort, P. J. I., Jacobsen, E., Visser, R. G. F., van der Vossen, E. A. G., Govers, F., and Vleeshouwers, V. G. A. A. 2009. *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. *Mol. Plant-Microbe Interact.* 22:1535-1545.
- Chen, Y., and Halterman, D. A. 2011. Phenotypic characterization of potato late blight resistance mediated by the broad-spectrum resistance gene *RB*. *Phytopathology* 101:263-270.
- Chen, Y., and Halterman, D. A. 2017a. Determination of virulence contribution from *Phytophthora infestans* effector IPI-O4 in a resistant potato host containing the *RB* gene. *Physiol. Mol. Plant Pathol.* 100:30-34.
- Chen, Y., and Halterman, D. A. 2017b. *Phytophthora infestans* effectors IPI-O1 and IPI-O4 each contribute to pathogen virulence. *Phytopathology* 107:600-606.
- Chen, Y., Liu, Z., and Halterman, D. A. 2012. Molecular determinants of resistance activation and suppression by *Phytophthora infestans* effector IPI-O. *PLoS Pathog.* 8:e1002595.
- Collier, S. M., Hamel, L.-P., and Moffett, P. 2011. Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein. *Mol. Plant-Microbe Interact.* 24:918-931.
- Collier, S. M., and Moffett, P. 2009. NB-LRRs work a "bait and switch" on pathogens. *Trends Plant Sci.* 14:521-529.
- de Jonge, R., Bolton, M. D., and Thomma, B. P. H. J. 2011. How filamentous pathogens co-opt plants: The ins and outs of fungal effectors. *Curr. Opin. Plant Biol.* 14:400-406.
- Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Teh, T., Wang, C. I., Ayliffe, M. A., Kobe, B., and Ellis, J. G. 2006. Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 103:8888-8893.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11:539-548.
- Eitas, T. K., and Dangl, J. L. 2010. NB-LRR proteins: Pairs, pieces, perception, partners, and pathways. *Curr. Opin. Plant Biol.* 13:472-477.
- Erickson, F. L., Dinesh-Kumar, S. P., Holzberg, S., Ustach, C. V., Dutton, M., Handley, V., Corr, C., and Baker, B. J. 1999. Interactions between tobacco mosaic virus and the tobacco *N* gene. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354:653-658.
- Farnham, G., and Baulcombe, D. C. 2006. Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proc. Natl. Acad. Sci. U.S.A.* 103:18828-18833.
- Giraldo, M. C., and Valent, B. 2013. Filamentous plant pathogen effectors in action. *Nat. Rev. Microbiol.* 11:800-814.
- Halterman, D. A., Chen, Y., Sopee, J., Berduo-Sandoval, J., and Sánchez-Pérez, A. 2010. Competition between *Phytophthora infestans* effectors leads to increased aggressiveness on plants containing broad-spectrum late blight resistance. *PLoS One* 5:e10536.
- Harris, C. J., Slootweg, E. J., Goverse, A., and Baulcombe, D. C. 2013. Stepwise artificial evolution of a plant disease resistance gene. *Proc. Natl. Acad. Sci. U.S.A.* 110:21189-21194.
- Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Karki, H. S., Jansky, S. H., and Halterman, D. A. 2021. Screening of wild potatoes identifies new sources of late blight resistance. *Plant Dis.* 105:368-376.
- Krasileva, K. V., Dahlbeck, D., and Staskawicz, B. J. 2010. Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22:2444-2458.
- Lokossou, A. A., Park, T. H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., Whisson, S. C., Birch, P. R. J., Visser, R. G. F., Jacobsen, E., and van der Vossen, E. A. G. 2009. Exploiting knowledge of *R/Avr* genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. *Mol. Plant-Microbe Interact.* 22:630-641.
- Mackey, D., Holt, B. F., 3rd, Wiig, A., and Dangl, J. L. 2002. RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell* 108:743-754.
- Mucyn, T. S., Clemente, A., Andriotis, V. M., Balmuth, A. L., Oldroyd, G. E., Staskawicz, B. J., and Rathjen, J. P. 2006. The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. *Plant Cell* 18:2792-2806.
- Ngou, B. P. M., Ahn, H.-K., Ding, P., and Jones, J. D. G. 2021. Mutual potentiation of plant immunity by cell-surface and intracellular receptors. *Nature* 592:110-115.
- Paulus, J. K., and van der Hoorn, R. A. L. 2018. Tricked or trapped-two decoy mechanisms in host-pathogen interactions. *PLoS Pathog.* 14:e1006761.
- Pieterse, C. M., Derksen, A. M., Folders, J., and Govers, F. 1994. Expression of the *Phytophthora infestans* *ipiB* and *ipiO* genes in planta and in vitro. *Mol. Gen. Genet.* 244:269-277.
- Rairdan, G. J., Collier, S. M., Sacco, M. A., Baldwin, T. T., Boettrich, T., and Moffett, P. 2008. The coiled-coil and nucleotide binding domains of the potato Rx disease resistance protein function in pathogen recognition and signaling. *Plant Cell* 20:739-751.
- Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., and Eliceiri, K. W. 2017. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* 18:529.

- Segretin, M. E., Pais, M., Franceschetti, M., Chaparro-Garcia, A., Bos, J. I. B., Banfield, M. J., and Kamoun, S. 2014. Single amino acid mutations in the potato immune receptor R3a expand response to *Phytophthora* effectors. *Mol. Plant-Microbe Interact.* 27:624-637.
- Shen, Q. H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I. E., and Schulze-Lefert, P. 2007. Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315:1098-1103.
- Song, J., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberland, G. T., Liu, J., Kuang, H., Austin-Phillips, S., Buell, C. R., Helgeson, J. P., and Jiang, J. 2003. Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *Proc. Natl. Acad. Sci. U.S.A.* 100:9128-9133.
- Stergiopoulos, I., and de Wit, P. J. 2009. Fungal effector proteins. *Annu. Rev. Phytopathol.* 47:233-263.
- van der Hoorn, R. A. L., and Kamoun, S. 2008. From guard to decoy: A new model for perception of plant pathogen effectors. *Plant Cell* 20: 2009-2017.
- van der Vossen, E., Sikkema, A., Hekkert, B., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., and Allefs, S. 2003. An ancient *R* gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. *Plant J.* 36:867-882.
- van der Vossen, E. A., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A., and Allefs, S. 2005. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. *Plant J.* 44: 208-222.
- van West, P., de Jong, A. J., Judelson, H. S., Emons, A. M. C., and Govers, F. 1998. The *ipiO* gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genet. Biol.* 23: 126-138.
- Wang, J., Hu, M., Wang, J., Qi, J., Han, Z., Wang, G., Qi, Y., Wang, H.-W., Zhou, J.-M., and Chai, J. 2019a. Reconstitution and structure of a plant NLR resistosome conferring immunity. *Science* 364:eaav5870.
- Wang, J., Wang, J., Hu, M., Wu, S., Qi, J., Wang, G., Han, Z., Qi, Y., Gao, N., Wang, H.-W., Zhou, J.-M., and Chai, J. 2019b. Ligand-triggered allosteric ADP release primes a plant NLR complex. *Science* 364: eaav5868.
- Wastie, R. L. 1991. Breeding for resistance. Pages 193-224 in: *Phytophthora infestans*, The Cause of Late Blight in Potato, *Advances in Plant Pathology*. D. S. Ingram, and P. H. Williams, eds. Academic, London.