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Presence of the Potato Late Blight Resistance Gene *RB* Does Not Promote Adaptive Parasitism of *Phytophthora infestans*

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ABSTRACT

The gene *RB* is derived from the wild potato species *S. bulbocastanum* and confers partial resistance to late blight, caused by the oomycete pathogen *Phytophthora infestans*. In order to investigate whether a single strain of *P. infestans* can adapt to overcome this partial resistance source, we subjected *RB* containing leaflets to multiple rounds of infection with *P. infestans*, with a culture isolated from a lesion used to infect the next leaflet (a passage). A parallel line of passages was done using susceptible leaflets as hosts. At the end of the experiment, *P. infestans* strains passaged through resistant or susceptible leaflets were compared for infection efficiency and lesion size. Variants of the *P. infestans* effector family IPI-O, some of which are recognized by the *RB* protein to elicit resistance, were cloned and sequenced to determine whether variation occurred during selection on the partially resistant host. Our results show that after 20 rounds of selection, no breakdown in *RB* resistance took place. In fact, the strain that was continually passaged through the partially resistant host produced smaller lesions on susceptible leaflets and had a lower infection frequency than the strain passaged through susceptible cultivar Katahdin. No changes within IPI-O coding regions were detected after selection on the hosts with *RB*. Our results indicate that individual strains of *P. infestans* are not capable of rapidly overcoming *RB* resistance even when it is the only host available.

Keywords: Host Disease Resistance; Pathogen Evolution; Partial Resistance; Pathogen Effector

1. Introduction

The oomycete phytopathogen *Phytophthora infestans* is the causal agent of late blight on potatoes (*Solanum tuberosum* L.). Late blight infections are particularly destructive and can spread rapidly throughout a field under favorable environmental conditions. Intensive long-term efforts have been made to identify and integrate host resistance into potato and have led to the identification of numerous resistance genes from wild potato species. Incorporation of race-specific genes from *S. demissum* initially provided protection against the disease, but increased popularity of resistant cultivars led to the selection of *P. infestans* genotypes that could overcome resistance [1-3]. Therefore, breeders have also focused on the development of cultivars with polygenic partial resistance in the hopes that it would be more durable [4-8].

Partial, or rate-reducing, host resistance results in reduced pathogen virulence as defined by decreased infection efficiency, diminished sporangia production, and a reduction in the size of necrotic lesions [9]. Although a promising source of long-term resistance, the durability of partial late blight resistance remains to be seen. Several reports have shown pathogen isolate specificity for partially resistant cultivars indicating adaptation that may result in the eventual breakdown of partial resistance [10-16]. However, the environmental conditions supporting adaptation to partially resistant cultivars remains uncertain since evidence suggests that pathogen populations are most likely to adapt to the most readily available host, regardless of environment or their resistance characteristics [15].

The wild potato species *S. bulbocastanum* has provided another source of resistance (*R*) genes that includes the gene *RB*, also named *Rpi-blb1* [17,18]. Cloning and initial testing of *RB* revealed that it was effective against a wide spectrum of *P. infestans* genotypes [19,20]. Plants containing this gene were also tested for several years in the Toluca Valley of Mexico, a hotspot for *P. infestans* diversity, with no breakdown in resistance [19]. The resistance response mediated by *RB*, which includes induction of programmed cell death, callose deposition, and

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increased transcription of pathogenesis-related defense genes, is similar to the response mediated by the immunity-conferring gene *R*9 from *S. demissum* [21]. However, *RB* only confers partial resistance and allows the growth and sporulation of *P. infestans* to a small degree [19,20, 22]. The partial resistance phenotype of plants expressing *RB* should not be confused with polygenic, partial resistance that has been described in several potato cultivars [13,15,16,23,24]. In these cases, several genes are thought to act together to provide an increased level of resistance whereas resistance mediated by *RB* acts in a gene-for-gene manner [25]. Therefore, it is logical to assume that adaptation by *P. infestans* to overcome or adapt to polygenic versus monogenic partial resistance would require different genetic mechanisms.

The *RB* protein recognizes the effectors IPI-O1 and IPI-O2 (class I), but not IPI-O4 (class III), and elicits resistance to *P. infestans* when class I effectors are introduced by the pathogen [26]. Previously, we have found that IPI-O4 suppresses IPI-O1-elicited resistance by *RB*, and isolates that contain IPI-O4 are more virulent on *RB* transgenic plants [27]. The number of IPI-O alleles and the presence or absence of IPI-O4 also varies be- tween isolates [26,27]. The fact that this locus is dynamic suggests the possibility of rapid evolution to obtain new variants of the effector, either through recombination or gene duplication within the IPI-O locus. Therefore, adaptive parasitism in *P. infestans* could occur due to growth on partially resistant leaves containing *RB*. If the pathogen is able to reproduce with subsequent generations living on the host, each generation of the pathogen may exhibit incrementally increased virulence against the formerly resistant host.

In the data presented here potato cultivar Katahdin containing a single copy of *RB* was exposed to multiple rounds of infection with *P. infestans*, with cultures isolated from a late blight lesion used to infect the next leaflet. A parallel line of inoculations was carried out using non-transgenic leaflets. At the end of the experiment, *P. infestans* strains passaged through resistant or susceptible leaflets were compared for their ability to cause disease. This was done to determine whether continued exposure to a host expressing *RB* would lead to local pathogen adaptation and a breakdown in resistance. Variants of IPI-O were also cloned and sequenced to determine whether variation occurred after selection on the partially resistant host.

2. Materials and Methods

2.1. Plant and Pathogen Materials

The plant materials used in these experiments were *Solanum tuberosum* cultivar Katahdin and the *RB* transgenic potato line SP951 (cv. Katahdin plus a single copy of *RB*) [22]. All potatoes were propagated from cuttings and maintained in the greenhouse, which was set for 18 h of daylight, a daytime temperature between 17˚C and 19˚C, and a nighttime temperature between 13˚C and 15˚C. *P. infestans* isolate US 940480 (US-8 genotype, A2 mating type) was maintained on solid Rye A media [28] in the dark at 15˚C. The original US8 isolate had been continually subcultured on Rye A media every 8 - 10 weeks for at least five years. Although US8 can cause some disease on *RB* plants, it is not considered a resistance breaking strain since plants with this *R* gene are clearly more resistant than those without [27,29,30].

2.2. Detached Leaf Infection Assays

The fourth to fifth leaf below the uppermost fully expanded leaf of six to eight week old Katahdin and SP951 plants was collected. Petioles were trimmed and leaflets were inserted into plastic boxes containing 0.67% water agar two to 24 hours before inoculating, with two leaflets per cube. *P. infestans* cultures (16 - 22 days old) on Rye A agar were flooded with sterile, distilled water and washes were combined to obtain a suspension of approximately 25,000 sporangia/ml. Sporangial suspendsions were placed at 12˚C for three hours to induce zoospore release. Leaflets were inoculated with 10 µl droplets on the abaxial side and kept at 15˚C in the dark. Eleven days after inoculation, the edges of sporulating regions were excised from both Katahdin and SP951 hosts. These sections were placed on Rye A media and clean cultures were subcultured once before inoculation. This procedure (a passage) was repeated multiple times, with each strain collected from Katahdin and SP951 used to inoculate the same host genotype. After the 20th passage, the Katahdin and SP951 strains (named US8-K20 and US8-SP20, respectively) were each used to inoculate both genotypes (cross-inoculation) using six 10 µl drops/ leaflet of 50,000 sporangia/ml for a total of 24 independent inoculations per strain/genotype interaction. Inoculations with the original US8, US8-K20, and US8-SP20 strains were repeated three times on different dates. Cubes were covered and placed in a 15˚C incubator for six days before reading results. Six days after the cross-inoculation, necrotic lesion diameters were measured in millimeters. The diameters were used to calculate mean lesion areas per inoculation from the 24 inoculation events. When present, chlorosis surrounding the inoculated areas extended beyond the necrosis, but was normally relative to the amount of necrosis present. Therefore, only necrosis was used to calculate lesion areas. Some necrotic lesions extended to the edge of the leaflet. In these cases, the longest diameter of the lesion was

used to calculate the area. Where no visible lesion was observed, the lesion area was calculated as zero. The infection efficiency was determined as the percentage of inoculated sites that developed sporulating lesions with an area greater than 1 mm^2.

2.3. IPI-O Cloning and Sequencing

The original US8 strain of *P. infestans* along with the strains that had been passaged through Katahdin and SP951 leaflets for 10 and 20 cycles were used for genomic DNA isolation. *P. infestans* strains were grown in liquid pea broth media and DNA was isolated using a previously published protocol [31]. Fifty nanograms of *Phytophthora* DNA were used as a template for polymerase chain reaction (PCR). Primers RD6F (5'-CGCATCGATGGTTTCATCCAATCTCAACACCGCCG - 3') and RD6R (5'-GATGCGGCCGCTATACGATGTCATAGCATGACA-3') were used to amplify IPI-O alleles using the following parameters: 94˚ for 1 min; 40 cycles of 94˚ for 15 s, 52˚ for 30 s, 68˚ for 1.5 min; 68˚ for 15 minutes. All amplifications were carried out using Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) with five nmoles of each primer. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturers instructions. At least ten plasmid clones containing *IPI-O* variants from each isolate were sequenced in both directions using vector-specific primers. Double-strand sequencing of DNA was carried out at the University of Wisconsin-Madison Biotechnology Center sequencing facility. Vector sequence removal and sequence analyses were performed using the Lasergene software package (DNASTAR, Madison, WI).

2.4. Data Analysis

All pathogen by host interactions were repeated at least 72 times with 24 technical replications within three biological replications. A one-way analysis of variance was performed on all data sets in order to separate means. All lesion area data are presented as means of all replications.

3. Results

3.1. Effect of Passaging on Infection Efficiency

In these experiments, *P. infestans* strain US8 was passed through 20 rounds of inoculations on leaflets of potato cultivar Katahdin or SP951. The resulting strains were named US8-K20 and US8-SP20, respectively. First, we observed whether the two strains passaged through susceptible or resistant leaflets differed in their ability to initiate infections. The majority of drop inoculations on SP951, which contains *RB*, did not result in the formation of a measurable necrotic lesion after six days. In contrast, the majority of inoculations on Katahdin produced large lesions (see below). A successful infection was defined as a necrotic lesion larger than 1 mm^2. On Katahdin leaflets, the percentage of infection spots increased from 58.3% for the original strain to 92.2% for strain US8-K20 and 88.2% for strain US8-SP20 (**Table 1**). On leaflets of SP951, the original US8 strain was able to form successful infections only 19.4% of the time. The US8-K20 strain that had been passaged through Katahdin increased its infection efficiency on SP951 to 35.6%. The US8-SP20 strain did not increase considerably in its infection efficiency and was successful in only 23.6% of the inoculations of SP951.

3.2. Virulence Changes Induced by Passaging

Strain virulence was measured by calculating the area of necrotic lesions six days after inoculation (**Table 2**). Unsuccessful infections were calculated as having an area of zero mm^2. The mean lesion size produced by the original US8 strain was 57.3 mm^2 on susceptible Katahdin leaflets compared to 0.9 mm^2 on partially resistant SP951 (**Figure 1**). Regardless of the strain used, lesions on the partially resistant host SP951 were smaller than on susceptible Katahdin ($P < 0.001$). The lesions of US8-K20 on Katahdin leaflets had a mean area of 222.0 mm^2 and were significantly larger than the lesions produced by the original US8 strain ($P < 0.001$). The mean lesion area of US8-K20 on SP951 leaflets was 4.4 mm^2, a significant increase over the original strain at $P = 0.033$.

Table 1. Percentage infection efficiency of *P. infestans* strains.

Strain	Katahdin			SP951		
	# lesions[1]	N[2]	% infection	# lesions	N	% infection
US8	42 ± 4.6	72	58.3%	14 ± 2.5	72	19.4%
US8 K20	71 ± 4.5	77	92.2%	26 ± 5.7	73	35.6%
US8 SP20	67 ± 5.1	76	88.2%	17 ± 3.2	72	23.6%

[1]Number of lesions with an area > 1 mm^2 ± the standard deviation between replicates; [2]N = total number of inoculation events.

Table 2. Lesion areas after drop inoculation on host leaflets.

Strain and host	Lesion area[1]	Lesion area[1] (lesions > 1 mm^2)
US8 on Katahdin	57.3 ±17.9	137.5 ± 19.4
US8 on SP951	0.9 ± 0.9	12.8 ± 7.8
US8-K20 on Katahdin	222.0 ± 38.7	255.2 ± 22.7
US8-K20 on SP951	4.4 ± 2.1	18.5 ± 3.7
US8-SP20 on Katahdin	156.5 ± 27.4	188.8 ± 16.2
US8-SP20 on SP951	1.7 ± 1.4	19.0 ± 6.6

[1]Lesion areas are shown in mm^2 ± the standard error.

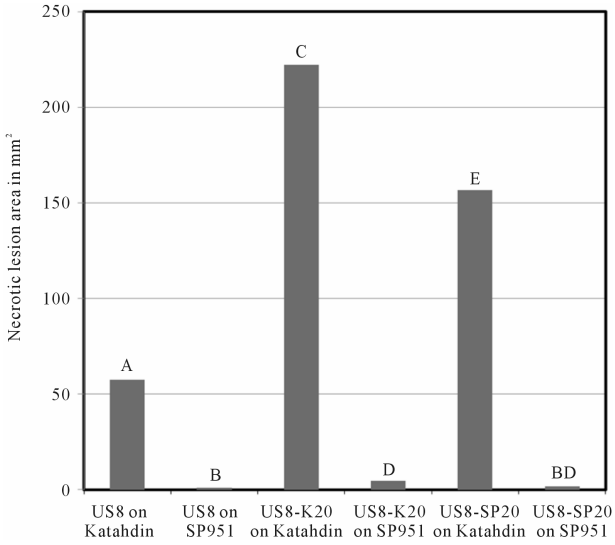

Figure 1. Mean lesion areas for all lesions. The original US8 strain as well as the strains passaged for 20 cycles through susceptible Katahdin (US8-K20) and partially resistant SP951 (US8-SP20) leaflets were drop inoculated on the hosts shown. Different letters above the bars denote significantly different means at P < 0.05.

Lesion areas of the US8-SP20 strain on the Katahdin host were 156.5 mm^2, which was significantly more than the original strain ($P < 0.001$) and less than the US8-K20 strain at $P = 0.011$. On SP951, the US8-SP20 strain had a mean lesion area of 1.7 mm^2, which was not significantly different from those of the original strain or US8-K20 on SP951.

Although the mean lesion area is a suitable basis for comparing virulence between strains, the decreased infection efficiency of some strains led to many lesions with no calculable area. Therefore, in order to account for decreased infection efficiency, the mean areas of infection produced by each strain were recalculated after removal of lesions less than 1 mm^2 (**Figure 2**, **Table 2**). In all cases, lesion areas on SP951 leaflets were significantly smaller than on Katahdin leaflets, regardless of the

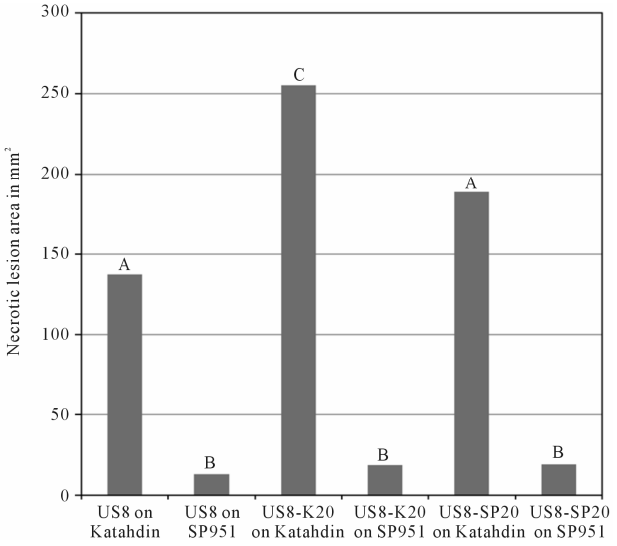

Figure 2. Means of lesion areas of successful infections. The original US8 strain as well as the strains passaged for 20 cycles through susceptible Katahdin (US8-K20) and partially resistant SP951 (US8-SP20) leaflets were drop inoculated on the hosts shown. Different letters above the bars denote significantly different means at P < 0.05.

strain used ($P < 0.001$). The removal of the non-infection events from the original US8 strain data led to an increase in the mean lesion area (137.5 mm^2). However, this was still significantly less than the mean area after it had been passaged through Katahdin leaflets (255.2 mm^2, $P < 0.001$), indicating that revival of the pathogen from Rye A media storage led to increased virulence in addition to enhanced infection efficiency. The difference between lesion areas of US8-K20 (255.2 mm^2) and US8-SP20 (188.8 mm^2) on Katahdin leaves remained significant at $P = 0.020$, demonstrating that the limited virulence of US8-SP20 is separate from it's ability to establish an infection. On SP951 leaflets, no significant differences in lesion areas were observed when comparing the three strains.

3.3. Analysis of IPI-O Coding Regions after Passaging

Sequencing of the coding region of IPI-O variants before and after selection on *RB* plants revealed that no selection for divergence had occurred within this gene family. The original US8 strain used for these experiments contained IPI-O1 and IPI-O2 variants [27]. After ten cycles of selection on partially resistant leaves, the isolate still contains only IPI-O1 and IPI-O2 variants of identical sequence. After twenty cycles, still no sequence variability was observed. After ten passages through susceptible Katahdin leaflets, one IPI-O2 variant was found that contained a single A to G nucleotide change at position

181 of the IPI-O coding sequence [27]. This nucleotide change resulted in a glutamate to glycine amino acid change at residue position 60 in the predicted protein. The original IPI-O2 sequence was also found in both of these isolates, suggesting that the mutation occurred in a duplicated gene sequence.

4. Discussion

The objective of this work was to observe the local adaptation of *P. infestans* on a host containing a single *R* gene conferring partial resistance. The US8 strain of *P. infestans* is capable of growth and sporulation on partially resistant potato leaves containing the *RB* gene [19, 20,22], but we have found it is not capable of completely overcoming the resistance response within 20 rounds of selection. Under ideal conditions, *P. infestans* can complete one generation (inoculation to sporulation) in 4 - 5 days [3,32]. Therefore, with a growing season of approximately 120 days, a field of potatoes under constantly ideal conditions for late blight would allow for 30 *P. infestans* generations. Since the environmental conditions in most potato-growing regions are not constantly suitable for late blight, we can deduce that our 20 rounds of selection might represent multiple years of exposure in the field.

We found that the presence of *RB* did not select for increased *P. infestans* virulence. In fact, the strain that was continually passaged through the partially resistant host produced smaller lesions on susceptible leaflets and had a lower infection frequency than the strain passaged through susceptible Katahdin. *P. infestans* has repeatedly proven its ability to overcome host resistance after an extended period of time [1-3]. It is generally thought that reduced virulence of *P. infestans* resulting from increased resistance in the host elicits pathogen adaptation due to the increased selection pressure. However, the pathogen's ability to adapt to overcome resistance is determined by the fitness penalty associated with the mutations that might occur. Therefore, one interpretation of our results would be that the ability of *P. infestans* to overcome the *RB* gene in the partially resistant SP951 plants results in a fitness penalty that can be observed after multiple generations of exposure to this resistance gene. One possible outcome of this interaction, therefore, could be a reduction in pathogen virulence after continual exposure to the *RB* gene. While proper testing of this hypothesis will require years of field testing, our results indicate that, at the very least, virulence of *P. infestans* strain US8 does not increase after exposure to the *RB* gene.

It has been known for quite some time that *P. infestans* that is continually cultured on artificial media (e.g. chick pea or Rye A) leads to decreased virulence [33]. We found no exception in our experiments since the original US8 isolate, which had been cultured exclusively on Rye A for at least five years, increased in infection efficiency dramatically after passage through potato leaflets. The difference in lesion areas between US8 and US8-K20 strains, even when unsuccessful infection events were removed, suggests that culturing for long periods on Rye A leads to decreased virulence. The increase in the percentage of successful infection events indicates an additional limitation in the ability of sporangia or zoospores to infect host cells and start an infection.

Our previous work with the *RB* gene suggests that the partial resistance in the host is not due to an inadequacy of the gene product to recognize or respond to *P. infestans*, since the suite of responses mediated by *RB* do not differ significantly from those mediated by the immunity-conferring gene *R*9 [21]. Rather, the ability of *P. infestans* to outgrow *RB*-induced responses appears to be due to a suppression mechanism that restricts specific defense responses after recognition [21]. Our data here supports this hypothesis since, when there is successful infection by *P. infestans*, the *RB* gene is effective at reducing but not stopping progression of the pathogen. Our results further suggest that the ability of *P. infestans* to suppress *RB* responses is associated with a fitness penalty since the US8-SP20 strain was less virulent than US8-K20. Whether this fitness effect is associated with expression of IPI-O variants or additional separate loci remains to be investigated.

Selection on partially resistant leaflets did not result in mutations leading to a dramatic increase in virulence (*i.e.* resistance breaking) such as has been observed in some isolates of *P. infestans* [27,34]. In the *RB* defeating isolates, the ability to completely overcome resistance is associated with the absence of class I or the presence of class III IPI-O variants [27,34]. After selection on *RB* leaflets, we did not observe alterations in IPI-O alleles that would explain changes in virulence. Therefore, the decrease in fitness that we observed does not appear to be due to variation in the coding sequence of IPI-O effectors. We cannot rule out the possibility, however, that changes in IPI-O expression might affect virulence since transcription or protein levels were not measured in these experiments. Transcription of IPI-O is strongly induced during the early stages of infection [35], which suggests a role in virulence or pathogenicity. Experiments to test the expression of IPI-O after exposure to *RB* are currently underway.

The *P. infestans* strain US8 passaged through susceptible Katahdin contained a nucleotide mutation that led to an amino acid change within one IPI-O variant. This amino acid site is not one that had been previously iden-

tified as being under diversifying selection [27] and it is not known whether this mutation is responsible for a change in virulence. This mutation is likely not due to amplification-based anomalies since multiple clones of this mutation were found and a high-fidelity, high-processivity polymerase was used to avoid the incorporation of random mutations. The IPI-O protein contains a RGD cell adhesion motif that binds to a lectin receptor kinase in Arabidopsis, which may function as an effector target [36]. This lectin receptor kinase mediates cell wall-plasma membrane adhesions, and IPI-O can disrupt these adhesions [37]. The role that this activity might play during infection is currently unknown.

Previous studies of *P. infestans* adaptation have focused on local populations and their interaction with the foliage of specific potato cultivars in the field [13,15,16, 23]. The results of these studies propose different possible outcomes for adaptation to partially resistant hosts. James and Fry [23] did not observe local adaptation to partially resistant hosts since changes in pathogenic ability of the populations were not specific for the host on which the population was developed. In contrast, several reports have shown that *P. infestans* is capable of adaptation for virulence of partially resistant foliage and tubers [10-12,38,39]. Work by Andrivon *et al*. [15] found that *P. infestans* populations adapted to the most locally dominant cultivar, regardless of the host resistance phenotype. All of these studies included hosts with unknown or polygenic resistance mechanisms, which would present different selective pressures on the pathogen compared to monogenic host resistance. Our focus on the specific interaction between an individual *P. infestans* strain and a single *R* gene is distinct from these previous reports and provides insight into the activity and potential durability of plants expressing only the *RB* gene. After 20 cycles of selection on leaflets with *RB*, an increase in pathogen virulence was observed when compared to the original US8 strain, due to restoration after continuous storage, but lesion size and infection efficiency were less than that of the strain exposed only to the wild-type Katahdin host. This clearly shows that *P. infestans* cannot adapt to the host genotype as easily in the presence of *RB*. Our experiments included a single US8 strain of *P. infestans*, which is the most dominant genotype in the US and is highly competitive against other genotypes [24]. To date, the *RB* gene has not been widely deployed making field-level trials difficult. However, future studies that include inoculated plots of potato with *RB* would help in ascertaining the potential durability of this *R* gene. When combined with other modes of action for late blight control (e.g. fungicide application, incorporation of other *R* genes), we would expect the addition of *RB* to be a valuable asset to any late blight control strategy.

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