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Evaluating the potential of *Phytophthora infestans* to genetically adapt to the Rpi-blb1 (RB) source of blight resistance

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SUMMARY
The *P. infestans* resistance gene *Rpi-blb1* is derived from the wild potato species *Solanum bulbocastanum* and the corresponding gene product recognizes the presence of the *ipiO* virulence effector of *P. infestans,* triggering a resistance response when this effector is introduced during invasion by *P. infestans*. The goal of this study was to compare the sequence variation in the *ipiO* gene following passage through detached leaves of Maris Peer (MP) expressing *Rpi*-blb1 with that resulting from passage through untransformed Maris Peer. After 10 repeat passages through MP + *Rpi-blb1*, 4 regions of the *ipiO* sequence were identified as containing up to 12 hotspots where sequence variants occurred at frequencies of between 1.79 % and 59.91%. Significantly, comparable levels of variation were also recorded following passage through untransformed MP - *Rpi-blb1* leaf tissue, suggesting that the *ipiO* sequence is in a steady state of flux as a result of being exposed to host tissue, irrespective of whether the host is equipped with the corresponding R gene or not.

KEYWORDS
Phytophthora infestans, Rpi-blb1, 454 sequencing, ipiO.

INTRODUCTION

P. infestans, like all plant pathogens, has to negotiate intricate defence systems in order to infect its host. During the biotrophic phase of *P. infestans* infection, the pathogen forms a structure called the haustorium with which it invaginates the host cell membrane, delivering pathogenicity factors ('effectors') into the host cytoplasm and acquiring nutrients from the host cell (Dodds and Rathjen, 2010). Plants may respond to this attack by attempting to restrict colonization, often through effector-triggered immunity which involves a form of programmed cell death

popularly known as the 'hypersensitive response'. To facilitate successful colonization, the *P. infestans* genome encodes effectors which operate inside the host cell (Haas *et al.*, 2009). The best known *P. infestans* effectors are those containing the RXLR (arginine-any amino acid-leucine-arginine) motif, which represents a conserved sequence determinant of host translocation (Birch *et al.*, 2006). Such effectors include the *ipiO* gene family which is highly diverse among *P. infestans* populations worldwide with class I IPI-O occurring in the majority of pathogen genotypes (Champouret *et al.*, 2009).

The wild potato *S. bulbocastanum* displays strong partial resistance to *P. infestans* mediated by *R* genes including the *RB* gene, also known as *Rpi-blb1* (Song *et al.*, 2003). The resistance response conferred by the *Rpi-blb1* gene includes the induction of a classical hypersensitive response and up-regulated transcription of pathogenesis-associated defence genes (Chen and Halterman, 2011). However, *Rpi-blb1* only confers strong partial resistance and can permit the growth and sporulation of *P. infestans,* albeit at significantly lower rates compared to the non-*Rpi-blb1* expressing control (Song *et al.*, 2003). The *Rpi-blb1* gene product recognizes the presence of the *ipiO* virulence effector of *P. infestans* and triggers a resistance response when this effector is introduced during invasion by *P. infestans* (Vleeshouwers *et al* 2008). The fact that the *ipiO* locus is prone to mutation (Haas *et al.*, 2009) suggests that in a scenario where *P. infestans* parasitizes and sporulates on a partially resistant host, each subsequent generation may exhibit increased virulence against the formerly resistant host.

To investigate whether continued parasitism on a host harbouring the *Rpi-blb1* gene would lead to pathogen adaptation and breakdown of resistance, this study experimented with a representative isolate of the recently occurring 6_A1 *P. infestans* genotype ('Pink 6'), which was passaged ten times through detached leaflets of transgenic potato plants carrying one copy of the *Rpi-blb1* gene. At the end of the repetitive passaging, levels of polymorphism within the *ipiO* sequence of each cycled isolate were assessed and compared with the initial isolate.

MATERIALS AND METHODS

Plant and pathogen preparation

The potato cultivar Maris Peer (MP) was used for this study along with an *Rpi-blb1*-expressing potato line of the same cultivar containing a single copy of *Rpi-blb1* (Wendt *et al.*, 2012). Plants were propagated from tubers under glasshouse conditions of 20^{o}C and a minimum of 16 h day length. For the passaging experiments, leaflets (fourth / fifth leaf) below the uppermost fully expanded leaves were collected from glasshouse maintained 6 to 8 week old plants. Three independent biological replicates per treatment were included in a single experiment. *P. infestans* isolate DL43B_A1 [SSR genotype 6_A1 (Pink 6), A1 mating type] was maintained on rye A media slants and subcultured every 4 months.

Passaging of P. infestans isolate DL34B_A1 through detached leaflets of Rpi-blb1-expressing transgenic potato

Prior to commencing, the isolate was firstly passed through leaflets of the susceptible cultivar, Kerr's Pink to restore any virulence lost in culture. Viable inoculum was produced 7 days post-inoculation by washing leaflets with sporulating lesions in 5 ml of sterile distilled water in 50 ml Falcon tubes to dislodge sporangia into suspension. The resulting suspensions were standardized with a haemocytometer to a concentration of 2 x 10^4 sporangia/ml and incubated at 4^{o}C for

2 hours to release zoospores. To conduct the first passage, inoculations were performed using inoculum prepared as described above. A single leaflet was deemed an experimental unit. The leaflets were placed lower surface uppermost in 9 cm diameter inverted Petri dishes with a layer of dampened paper towel at the base before being inoculated in the centre with 20 µl of the appropriate sporangial/zoospore suspension. Inoculated leaflets were incubated in a growth chamber at 18^{o}C for 7 days with a 16 h photoperiod. For mean area under lesion progress curve (AULPC) assessment visible lesion area was calculated for each inoculated leaflet as 1/4πab for area of an ellipse with a = breadth of lesion and b = lesion length (Colon *et al.*, 1995). Lesion diameter along the main leaf vein and diameter perpendicular to the main leaf vein were deemed length and breadth respectively for consistency. Seven days post inoculation leaflets of each isolate/cultivar interaction were washed separately in sterile 50 ml Falcon tubes containing 2.5 ml of sterile distilled water to obtain inoculum for the next passage. As the same leaves were used for lesion area measurements and for inoculum production for the next passage, caution was observed to avoid the cross contamination among samples at all stages of the experiment. The same procedure for inoculum preparation and leaflet inoculation was followed for ten consecutive cycles. Cycle 0 was *P. infestans* inoculum that was not exposed to MP leaf tissue. Pure cultures were obtained upon completion of the tenth cycle by transferring sporangia from sporulating lesions individually onto pea agar without antibiotic. Three plates were prepared for each isolate during transfer of sporangia onto pea agar to reduce the risk of loss of isolates through contamination.

DNA extraction, ipiO amplification and 454 sequencing

Approximately 50 mg of *P. infestans* mycelia were harvested from 10 day old pea agar cultures of isolates using sterile scalpels and transferred into 2 ml Eppendorf tubes. The mycelia were freeze dried for 24 h and pulverized using a mixer mill with sterile glass beads. Extractions were performed using the procedure of Raeder and Broda (1985).

To determine the level of polymorphism within the ipiO gene, the target sequence was firstly PCR amplified under conditions of 0.2 mM dNTPs, 1X thermal buffer, 100 nM forward primer, 100 nM reverse primer, 1 unit Taq polymerase, 50 ng/µl genomic DNA and 13.8 µl water in a total volume of 20 µl. The reactions were completed in a Biometra 3500T Thermo Cycler with an initial cycle of denaturation of 5 min at 95°C followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 58°C and extension for 40 sec at 72°C and a final extension for 5 min at 72°C. The *ipiO* primers used were: 5' ATG GTT TCA TCC AAT CTC and 5' CTA TAC GAT GTC ATA GCA TGA CAC. PCR products were loaded onto 1% (w/v) agarose gel stained with ethidium bromide (1 µg/ml) and submerged in 0.5 M TBE buffer. A size standard (100 bp ladder NEB, UK) was loaded alongside the samples and 70 V of current applied for 30 min. Bands were visualized and imaged using a Kodak Imager (Image Station 440 CF, Kodak Digital Science TM, USA). Once identified, the requisite bands were excised with a scalpel and transferred into sterile 1.5 ml tubes. DNA was eluted using the Qiaex II Kit (Quiagen, Germany) following the manufacturer's instructions. The resulting supernatant containing the purified amplicons from each sample was transferred into fresh 1.5 ml Eppendorf tubes and quantified using a fluorescent Qubit DNA Assay (Invitrogen, USA). Generated amplicons were sequenced on a Roche GS Junior 454 sequencing platform according to manufacturer's recommendations for sample preparation and run completion. Data analysis was performed using the Roche proprietary AVA™ software, to analyse variant frequency. Variants were selected as positive when they dominated in >2% of the reads collected per *P. infestans* sample.

RESULTS AND DISCUSSION

Disease progression

For the conventional Maris Peer DL34B_A1 host pathogen interaction, disease progressed steadily from cycle 1 (AULPC = 647.19 +/-24.20) up to cycle 10 (AULPC = 1105.67 +/-27.52). In the case of the MP+*Rpi-blb1* line (equipped with a single copy of *Rpi-blb1* transgene), an initial AULPC score of 54.27 +/-4.14 was recorded after the first infection cycle. Notably, the disease lesions caused by DL34B_A1 enlarged progressively in subsequent cycles of inoculations (data not shown), with an AULPC of 1003.65 +/-44.82 recorded at cycle 10. One possible explanation to account for the occurrence of disease on RB-bearing hosts is delayed or ineffective triggering of the hypersensitive response (HR) (Vleeshouwers *et al.*, 2000) and/or the positioning of the integrated *Rpi-blb1* transgene within the Maris Peer genome. As the *Rpi-blb1* confers strong partial resistance in the form of a disease-rate-reducing phenotype which is different from immunity (Song *et al.*, 2003), delayed or ineffective HR is inevitable and allows escape and proliferation of pathogen hyphae which are necessary for establishment of the biotrophic phase of parasitism (Vleeshouwers *et al.*, 2000).

IpiO sequencing

Diversity across the *ipiO* sequence was assessed for nine DL34B_A1 *P. infestans* samples; that is from the three replicates of cycle 0, the three replicate isolates passaged through the MP + *Rpi-blb1* line and the three replicate isolates passaged through the conventional Maris Peer control for 10 cycles. The number of reads ranged from 5,356 to 8,662, with an average read number per template of 7,070 +/-415. As a result, a consensus of the *ipiO* sequence before exposure to potato leaf tissue was prepared from 19,172 reads (cycle 0) (Table 1), which acted as the reference sequence with which to compare against the *ipiO* reads obtained after the 10 repeated passages through the MP + *Rpi-blb1* material.

Table 1. *Number of 454 reads per individual P. infestans DL34B_A1 isolate before (cycle 0) and after (cycle 10) passaging through MP / MP + Rpi-blb1 potato leaf tissue*

Host	Cycle	Replicate	No. 454 reads	Mean (+/- SD)
MP	10	1	8337	--
MP	10	2	8662	---
MP	10	3	5945	7648 +/- 856
MP + Rpi-blb1	10	1	7711	---
MP + Rpi-blb1	10	2	7910	---
MP + Rpi-blb1	10	3	5895	7172 +/- 641
---	0	1	7789	---
---	0	2	5356	---
---	0	3	6027	6390 +/- 725

Following 10 successive cycles on MP + *Rpi-blb1* material the *P. infestans* sequence underwent substantial genetic change with a comparative analysis of each replicate's batch of reads against the respective *ipiO* consensus sequence (from cycle 0) indicating the prevalence of 12 'hotspots'

across approximately 4 regions of the *ipiO* sequence (90bp, 163 – 167bp; 278 – 304bp and 340 – 368bp) (Table 2).

Table 2. *Percentage sequence variation in the ipiO gene in three replicate P. infestans DL34B_A1 isolates (R1, R2 and R3) after 10 cycled passages through MP / MP + Rpi-blb1 potato leaf tissue*

Variant No.	bp position	MP			MP + Rpi-blb1		
		R1	R2	R3	R1	R2	R3
1	90	59.91	57.01	57.19	53.09	56.8	57.03
2	163	2.33	3.02	0.98	2.97	2.57	2.73
3	167	2.83	3.47	1.80	3.67	3.2	3.2
4	278	13.45	12.73	13.49	14.62	31.61	13.87
5	289	13.46	12.78	13.49	14.61	31.68	13.87
6	290	13.47	12.77	13.49	14.61	31.68	13.87
7	304	13.34	12.66	13.41	14.54	31.54	13.81
8	340	13.46	12.72	13.47	14.55	31.62	13.73
9	343	4.16	4.06	2.07	1.79	1.93	4.13
10	352	20.56	18.71	17.80	15.81	21.86	18.79
11	367	6.00	6.48	6.64	8.94	13.65	7.22
12	368	20.69	20.19	21.68	22.82	47.44	21.14

Variant frequency varied across the population, ranging from 1.93% to 59.91%, with variant no. 1 occurring in an average of 58.03% of *ipiO* sequences following exposure to leaf tissue expressing *Rpi-blb1* transcripts. While this contrasts with a recent study which concluded that the presence of the *RB* sequence did not promote adaptive parasitism in *P. infestans* (Halterman and Middleton, 2012), this report relied on direct sequencing of cloned *ipiO* amplicons which is not comparable to the coverage obtained through the 454 platform. Of significance for this study, however, was the comparable level of variation recorded across the *ipiO* sequence following passage through the control, non-transgenic plant material, which is not equipped with the *Rpi-blb1* transgene. While the level of variant occurrence was variable, the same variants were recorded irrespective of whether the infecting *P. infestans* isolate was passaged through host leaves with and without the *Rpi-blb1*resistance. This result was unexpected owing to the specific gene-for-gene interaction that exists between *ipiO* and the *Rpi-blb1* gene from *S. bulbocastanum*. In fact it was hypothesised that the absence of the R gene in the corresponding host would not result in any genetic change in the corresponding effector coding sequence in *P. infestans.* Based on the results presented here, this does not appear to be the case as the *ipiO* effector sequence in *P. infestans* is registering a constant state of flux during the potato – *P. infestans* host-pathogen interaction.

CONCLUSIONS

Employing a next generation sequencing approach, this preliminary study indicates the substantial potential for sequence change in an effector coding sequence after successive passages through clonal host tissue, irrespective of whether the potato leaf tissue was expressing the corresponding R gene. Further experimentation is required with additional genotypes of *P. infestans* to ensure the observed phenomenon is not genotype-specific and also

to investigate the impact of this repeat passaging on the sequence integrity of other effector coding sequences in the *P. infestans* genome.

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