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Gene *Rpi-bt1* from *Solanum bulbocastanum* Confers Resistance to Late Blight in Transgenic Potatoes

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Abstract *Phytophthora infestans*, the causal agent of late blight is the most devastating pathogen of cultivated potato worldwide. Utilizing map based cloning; a genomic region containing a cluster of six nucleotide binding site-leucine-rich repeat resistance gene analogs was isolated from the wild potato species *Solanum bulbocastanum*. Four genes were pseudogenes, with coding sequences interrupted by either frame shift mutations or premature stop codons. However, neither of the two uninterrupted genes conferred resistance to *P. infestans* when introduced into susceptible potatoes. Specific primers for one of the pseudogenes were used to amplify an uninterrupted cDNA from *P. infestans*-infected *S. bulbocastanum* leaves. A corresponding gDNA was amplified from a late blight-resistant *bulbocastanum–tuberosum* introgression line (*Rpi-bt1*). The *Rpi-bt1* gene under transcriptional control of the constitutive potato *Ubi3* promoter was found to confer resistance to *P. infestans* in several transgenic potato lines in a whole plant greenhouse assay.

Resumen *Phytophthora infestans*, el agente causal del tizón tardío, es el patógeno mas devastador de la papa cultivada en el mundo. Mediante la clonación basada en mapas, se aisló de la especie silvestre de papa *Solanum bulbocastanum* una región genómica que incluía un grupo de repetición con seis análogos de genes de resistencia de sitios de unión de nucleótidos ricos en leucina. Cuatro genes fueron pseudogenes, con secuencias de codificación interrumpidas ya fuera por mutaciones por cambio de marcos o por codones de terminación prematura. No obstante, ninguno de los dos genes sin interrupción confirió resistencia a *P. infestans* cuando se introdujeron a papas susceptibles. Se utilizaron iniciadores específicos para uno de los pseudogenes para amplificar un ADNc ininterrumpido de hojas de *S. bulbocastanum* infectadas con *P. infestans*. También se amplificó un ADNg correspondiente de una línea de introgresión (*Rpi-bt1*) de *bulbocastanum-tuberosum* resistente al tizón tardío. Se encontró que el gen *Rpi-bt1* bajo control transcripcional del promotor de papa *Ubi3* confiere resistencia a *P. infestans* en varias líneas de papa transgénica en un ensayo con planta completa bajo invernadero.

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Abbreviations

NBS-LRR	Nucleotide Binding Site-Leucine-Rich Repeat
RACE	Rapid Amplification of cDNA Ends
BAC	Bacterial Artificial Chromosome
-ps	pseudogene

Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most destructive plant diseases worldwide. *P. infestans* was the causative agent of the Irish Potato Famine of 1846-1850. Recently, late blight has been effectively controlled by the use of fungicides. However, late blight has become an increasingly important problem to agriculture in the United States and many other countries in the past decade, as more aggressive fungicide-resistant and host-specialized isolates of *P. inf*estans have appeared.

Breeding for resistance represents an obvious alternative to fungicide application. Dominant vertical resistance (R) genes from *S. demissum* formed the basis of potato breeding against *P. infestans* from the 1930s to 1950s (Black et al. 1953). While eleven vertical R-genes from *S. demissum* have been introgressed into cultivated potato (Gebhardt and Valkonen 2001; Malcolmson and Black 1966), these race-specific vertical R-genes were found to be non-durable and rapidly overcome when introduced into cultivars (Nelson 1978). In contrast to vertical resistance, horizontal resistance generally involves multiple genes and provides a more general, stable, pathogen resistance in a race-nonspecific manner. Horizontal resistance is not correlated with the hypersensitive response, and instead involves limiting pathogen spread in the host. *S. bulbocastanum,* for example, contains a dominant R gene locus apparently conferring horizontal resistance to *P. infestans* when introgressed into the cultivated potato (Naess et al. 2000; Naess et al. 2001). A gene named *RB* (also known as *Rpi-blb1*) has since been isolated from the *RB* resistance locus of *S. bulbocastanum* and found to confer broad spectrum resistance to *P. infestans* when introduced into potato (Song et al. 2003; van der Vossen et al. 2003).

The *RB* locus consists of a cluster (Meyers et al. 2005) of nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins, similar to most previously identified R genes (Martin et al. 2003). Here we report map-based cloning of the *Rpi-bt1* from the *Rpi-bt* locus of *S. bulbocastanum* isolated from the *bulbocastanum- tuberosum* (*bt*) introgression. *Rpi-bt1* is a NBS-LRR R-gene, with high similarity to *RB*, which in transgenic potatoes confers a high level of resistance against *P. infestans*.

Materials and Methods

Plant Material

Solanum bulbocastanum and *S. tuberosum* cvs. Lenape (Akeley et al. 1968) and Atlantic (Webb et al. 1978) were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin. Plants were maintained in tissue culture and grown in a glasshouse in Albany, CA.

For mapping, the back-cross 2 (BC2) *P. infestans* resistant clone J101k6A22 (Naess et al. 2001) was crossed with the Aberdeen, ID breeding selection A86102-6 (Joseph Pavek, unpublished) to generate the A9510 back-cross 3 (BC3) population. The BC3 population consisting of 100 progeny segregating for *P. infestans* resistance (scored Resistant, Susceptible, or Resistant/Susceptible) was employed in the present study.

Disease Assay

Detached leaf assays for late blight resistance employed unblemished, fully expanded sub terminal leaflets from 6 to 8 week old greenhouse-grown test plants. Inocula were obtained from two-week-old cultures of *P. infestans* (strain US8, Florida isolate) grown on rye agar. Inoculations were made by placing a 10 μL droplet of a sporangial suspension (4×10^4 mL^{-1}) that had been incubated at 8 C for 2.5 h (to release zoospores) on both sides of the midrib of the abaxial leaf surface. The inoculated leaflets were placed in Petri dishes containing moistened filter paper to maintain 100% relative humidity. Inoculated material was incubated for 1 d at 15 C in darkness, then for 4 d at 15 C with 6 h/d photoperiod (400 Em^{-2} s^{-1}). A computer-driven image analysis system (Bioquant IV, R and M Biometrics, Nashville, TN) was used to obtain measurements of lesions. The lesion diameter was determined by projecting the whole leaves onto a grid lining the Bioquant Digitizing Pad. The digitizing pad was coupled with an IBM PC and measurements were generated using Bioquant Systems software.

A whole-plant glasshouse test (Stewart et al. 1983) was used to determine which of the transgenic lines containing the genomic *Rpi-bt1* transgene (described below) were resistant to *P. infestans*. Tubers were planted in 15-cm pots and grown in a 16 h light and 8 h dark photoperiod using high-pressure sodium lamps as supplemental lighting. Inocula were obtained from cultures of *P. infestans* (strain MD-02-pet-1 A2, US-8 genotype) grown on lima bean agar medium in the dark at room temperature. After two weeks of incubation, the plates were flooded two times with sterile water and scraped lightly using an L-shaped glass or plastic rod to collect sporangia. The liquid from the plates were filtered into a 1 L glass beaker using two

layers of cheesecloth. The total volume was roughly estimated and sporangia concentration was determined using a hemacytometer. Using sterile water, the volume of the inoculum was adjusted to give a final concentration of 5×10^3sporangia mL^{-1}. The inoculum was transferred into a sprayer (approximately 2 mL sec^{-1}) after incubation at 4 C for 1 h followed by room temperature incubation for 30 min. Plants of each clone (in flower bud stage) were inoculated with *P. infestans*. Each plant was scored daily using Malcolmson's scoring scale (1–9) for increasing resistance (Cruickshank et al. 1982), starting 7 days after inoculation, and plants of each clone were compared.

AFLP and RFLP Mapping

The BC3 population segregating for *P. infestans* resistance was mapped using a combination of AFLP (Vos et al. 1995) and RFLP markers. AFLP mapping employed the commercially available primer/enzyme sets (Applied Biosystems, Inc.). The late blight resistance locus has been mapped to chromosome 8 (Naess et al. 2001). RFLP mapping employed tomato markers kindly provided by S. Tanksley (CT64, CT214, TG385, TG478, TG505, CD60, and TG282). Markers were amplified using M13 forward and reverse primers and random primed (GE Healthcare) [^{32}P]-labeled sequences were used to probe genomic blots of the BC3 parents. After the initial parental screening to identify polymorphisms, selected RFLP markers known to be located on potato chromosome 8 were also mapped in the BC3 population.

BAC Library Screening

The RFLP markers CT64, TG282, TG505, and CD60 were used to probe a *S. bulbocastanum* BAC library (Song et al. 2000). The library was probed with the random primed (GE Healthcare) [^{32}P]-labeled sequences. BAC clones corresponding to the RFLP markers were isolated and end-sequenced with M13 forward and reverse primers. These and subsequent BAC end-sequences were used to generate specific primer pairs (see Table 1 for the CD60 contig primers) for screening of pooled BAC clones by PCR to assemble contigs of overlapping BAC clones (Cai et al. 1995). To assess linkage of BACs to the resistance phenotype, BAC end sequence primer pairs were also employed in PCR screening of genomic DNA from the original population segregating for late blight resistance.

Isolation of Candidate Genes

Using BAC end and synthetic primer sequencing the *Rpi-bt* locus was localized to BAC clone C29F2F3 of the CD60 contig. Portions of the *Rpi-bt* locus were sequenced by digestion of BAC C29F2F3 and C29F2F2R2 and synthetic primer sequencing of the subcloned fragments. Three contigs covering approximately 75 kbp of the *Rpi-bt* locus were assembled. Genomic fragments containing each of the 6 complete NBS LRR coding sequences were obtained by restriction enzyme digestion of BACs C29F2F3 and C29F2F2R2. The *Rpi-bt1-ps* was isolated as an 11 kbp SphI-PacI fragment; and *Rpi-bt2* was isolated as a 10 kbp SphI-SacII fragment from C29F2F2R2. *Rpi-bt3* was isolated as an 18 kbp SacII-BamHI fragment; *Rpi-bt4-ps* and *Rpi-bt5-ps* were isolated on a 34 kbp SacII-PmeI fragment; and *Rpi-bt6-ps* was isolated as a 24 kbp PacI-PmeI fragment from C29F2F3. A genomic DNA for *Rpi-bt1* was obtained by PCR amplification using the DNA of a late blight-resistant BC3 line as a template and *Rpi-bt1-ps* specific primers (forward CCAACATCTTACTTCATTTCA and reverse GATACCGTTAGCTATAAGAC).

Sequences were compared to the GenBank non-redundant database using the BLAST Network Service of the National Center for Biotechnology Information (Altschul et al. 1990). Pustell matrix analysis (Pustell and Kafatos 1982) was used to compare DNA sequences and generate alignment presented in the figures (MacVector 8.0).

Identification of Transcripts from the *Rpi-bt* Locus

To identify potential mRNAs from *Rpi-bt* genes, RACE (Rapid Amplification of cDNA Ends)-PCR was employed using polyA$^+$ RNA prepared from *P. infestans*-infected *S. bulbocastanum* leaves. Transcript cDNAs were generated using the Marathon cDNA Amplification Kit (Clontech) followed by PCR amplification with gene specific primers for *Rpi-bt2* (5'RACE GGCATACAAGTCAATCCATGG CAACG and 3' RACE ACAGAGAGACAAGTTGCTA CACGCC) and *Rpi-bt3* (5'RACE GAATGCTACTGCCA CGCAAGTTCAAC and 3' RACE TTCCCTGAGGCT GAGTTATCATCACC). A messenger RNA transcript corresponding to the pseudogene (-ps) *Rpi-bt1-ps* was amplified with *Rpi-bt1-ps* specific primers (5'RACE AGT GATTCATCCAGTCTGGGAGACG and 3' RACE CCAAGCTGTGCTAGAAGATGCTCAG) from a late blight resistant BC3 line (*Rpi-bt1*).

Confirmation of the *Rpi-bt1* Gene Source

To confirm the source of the *Rpi-bt1* gene PCR was employed using genomic DNA from the backcross 1 and 2 parents ('Katahdin' and 'Atlantic') and *S. bulbocastanum*. PCR fragments were generated with forward primer (CCAAGCTGTGCTAGAAGATGCTCAG) in the first exon and reverse primer (GGCTTTATCCTGTCATTTA GACTTGGGG) in the first intron. PCR fragments were

Table 1 BACs and primers used for mapping the NBS-LRR locus shown in Fig. 2. Probes were amplified from 5′/3′ primer pairs for BAC end sequence derived from M13 forward or reverse primers

BAC	F/R[a]	Primer Sequence	Tm (ºC)[b]	Product (bp)
C29	F1 5′	CCCAAACGCCAAACCATA	53.7	
	F2 3′	AAGCTTAAGTTGTTCAAGTTTTC	53.5	201
	R1 5′	GAAACCCTAAAAAACTTGATCC	54.7	
	R2 3′	GACGCGACTTAAAGTCGT	53.7	396
C29F2	F1 5′	CTCAAAAGTAGCATTAAGCTAG	54.7	
	F2 3′	TGTGATCATTTAGCATTGACC	54.0	154
	R1 5′	AGCTTGACTTGATGCAGG	53.7	
	R2 3′	TCAAATCACCCCACTCTG	53.7	141
C29F2F2	F1 5′	GAATCCATCAATACGACAAC	53.2	
	F2 3′	CTTTGCTCTAATATAGGGAG	53.2	203
	R1 5′	AGCTTTGACATGTTCCCC	53.7	
	R2 3′	GCCAAATGACATGTGCAG	53.7	273
C29F2F2R1	F1 5′	CTGATAACAGAAGAAATGCTG	54.0	
	F2 3′	CTCAATAACTGAATACTCCTC	54.0	165
	R1 5′	GGAGAATAAGAAGGCACC	53.7	
	R2 3′	GGATGGCACATGGTTTTC	53.7	274
C29F2F2R1F3	F1 5′	GCTTGATGAAAGAGGTCG	53.7	
	F2 3′	TGGTGTACTCCCACAAGT	53.7	210
	R1 5′	CTTTCACCGCCAAGTTTG	53.7	
	R2 3′	CTGGAAGTAGTCTCTAGC	53.7	351

[a] Location of probe and primer orientation based on M13 forward and reverse primer BAC end sequencing

[b] Primer T_m calculation (69.3+0.41*%GC-650/#bp)

subjected to EcoRI restriction enzyme digestion to confirm the presence of the unique EcoRI site in the *S. bulbocastanum* BAC DNA and the BC3 gDNA isolates.

Screening of Candidate Genes for *Phytophthora* Resistance

Experiments to determine the efficacy of either *Rpi-bt2* or *Rpi-bt3* in conferring late blight resistance were based on mobilization of these genes plus at least 3 kbp of 5' and 3' flanking sequence into susceptible potatoes by *Agrobacterium*-mediated transformation. The 10 kbp *Rpi-bt2* fragment and the 18 kbp *Rpi-bt3* fragment were mobilized into the binary transformation vector pCGN1547 (McBride and Summerfelt 1990). These binary vector constructs were used to introduce the *Rpi-bt2* or *Rpi-bt3* genes into Lenape (Akeley et al. 1968) and Atlantic (Webb et al. 1978) by a standard transformation/selection protocol (Snyder and Belknap 1993). Transgenic and control lines were screened for *P. infestans*-resistance by the detached leaf assay (described above).

In order to express *Rpi-bt1* in transgenic plants a chimeric transgene was constructed. Transcription of the genomic copy *Rpi-bt1* gene was placed under the control of the potato *Ubi3* promoter for constitutive moderate-level expression (Garbarino and Belknap1994a; Garbarino and Belknap 1994b). The *Ubi3* polyadenylation signal was fused to the 3' end. The *Rpi-bt1* transgene was mobilized into the binary transformation vector pBINPLUS/ARS (Belknap et al. 2008; McCue et al. 2006). This binary vector construct was used to introduce the *Ubi3::Rpi-bt1* transgene into Atlantic. Transgenic and control lines were screened for *P. infestans*-resistance in the detached leaf and whole plant assays (described above).

Results and Discussion

Mapping of the *P. infestans* Resistance Locus

Because *S. bulbocastanum* cannot be crossed with potato, DNA from this diploid species was originally introgressed into *S. tuberosum* cv. Atlantic (Webb et al. 1978) by somatic fusion at the University of Wisconsin resulting in the somatic hybrid J101 (Naess et al. 2001). This somatic fusion was then backcrossed to 'Katahdin' resulting in the back-cross 1 (BC1) population. The resistant individual J101K6 from this BC1 population was then again crossed with Atlantic, resulting in the BC2 population containing the resistant clone J101k6A22 (Naess et al. 2001). This

BC2 clone was crossed with the 'Aberdeen', ID breeding selection A86102-6 (Joseph Pavek, unpublished) to generate the A9510 BC3 population employed in the mapping studies. This BC3 population consisted of 100 progeny segregating for *P. infestans* resistance. A combination of AFLP (Vos et al. 1995) and RFLP markers was employed to map the late blight resistance gene locus. The late blight resistance locus was previously mapped to chromosome 8 (Naess et al. 2001). The segregating population was subjected to AFLP mapping. Exhaustion of the commercially available AFLP primer/enzyme sets resulted in identification of over 400 polymorphic bands. RFLP mapping of the segregating population identified two markers, CT64 and TG505, that produced polymorphic bands that link with the R phenotype. The relative positions of the AFLP and RFLP markers closest to the *Rpi-bt* locus are shown in Fig. 1. AFLP analysis failed to generate multiple markers in the proximity of the RFLP probes, potentially due to the limited size of the BC3 population. A *Phytophthora resistance locus* was previously mapped between TG261 and CT88 (Naess et al. 2001). In tomato three genes, CD60, TG282, and TG505 are tightly linked with TG261 and the four genes map on the same locus (Tanksley et al. 1992). In potato CD60 lies in a region within 5.3 cM of TG261 (Tanksley et al. 1992). The clustering of these markers, together with the absence of multiple AFLP markers in the proximity of clustered RFLP probes suggested that additional mapping was unnecessary, and the RFLP markers were selected to probe a *S. bulbocastanum* BAC library (Song et al. 2000).

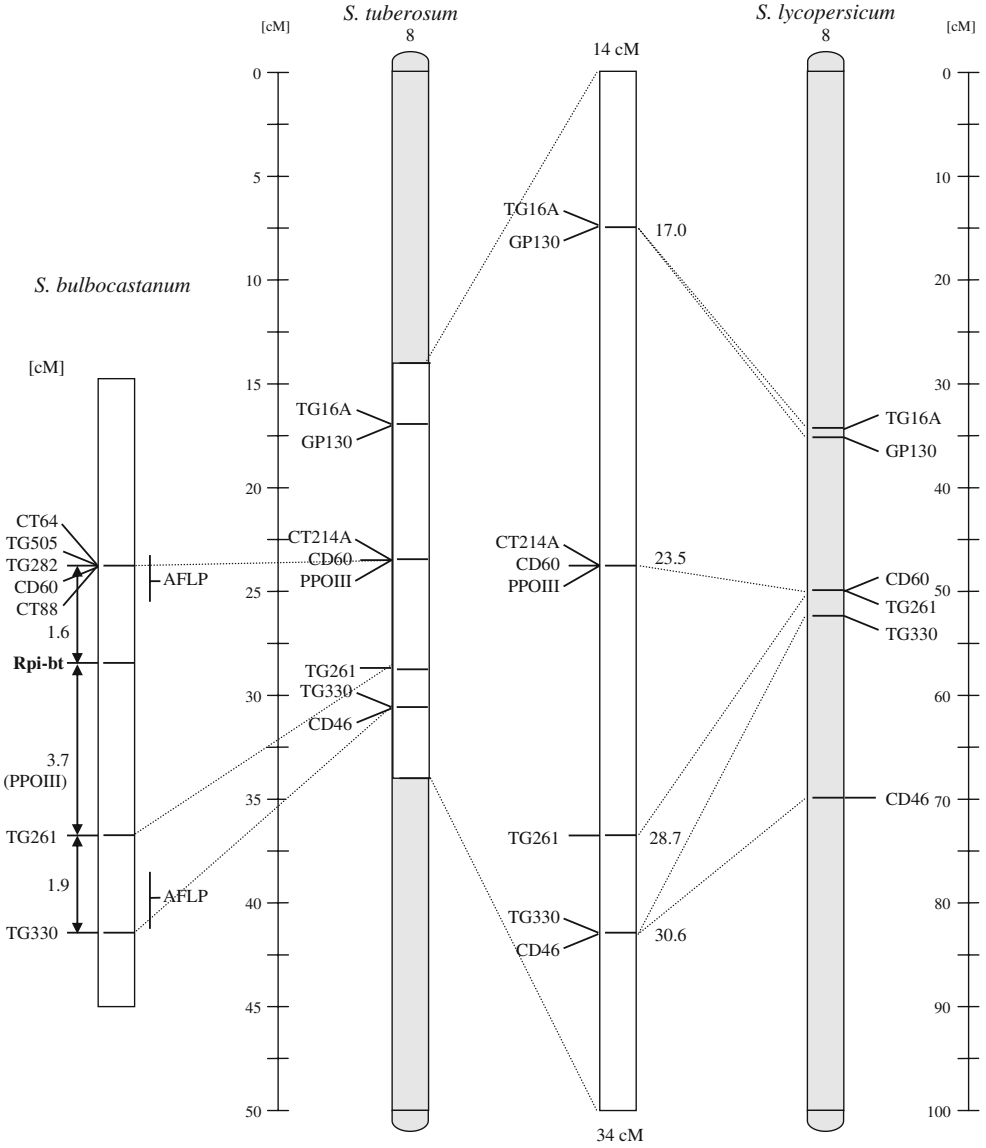

Fig. 1 The genetic map of the *S. bulbocastanum* late blight resistance gene locus compared to genetic maps of *S. tuberosum* and *S. lycopersicum* chromosome 8. The approximate position of the locus is indicated by Rpi-bt. The positions of RFLP markers relative to this locus are indicated. The relative positions of AFLP markers flanking the R gene are indicated. Data are from (Naess et al. 2001; Tanksley et al. 1992) adapted from the SOL Genomics Network (http://www.sgn.cornell.edu)

Identification of Candidate Resistance Genes

BAC C29 was originally identified by hybridization of library filters to the labeled CD60 RFLP marker. BAC end-sequence data from the M13 forward and M13 reverse primers (F and R) allowed design of specific primer pairs for both ends of each insert (Table 1). To obtain adjacent overlapping BACs (chromosome walking) subsets of the BAC library were pooled and screened by PCR using the end-specific primer sets (Cai et al. 1995). After six rounds, an approximately 700 kbp contig was assembled proximal to the CD60 RFLP marker on *S. bulbocastanum* chromosome 8 (Fig. 2). Using BAC end-sequence primer sets, BACs C29F2 and C29F5 were found to be linked to the resistance phenotype by PCR screening with BAC end sequence primer sets on genomic DNA from the original population segregating for late blight resistance. Computational (BLAST) alignment of the R end sequence of BAC isolate C29F2F2R2 with the GenBank non-redundant database revealed the position of a member (*Rpi-bt3*) of a NBS-LRR protein similar to many previously identified R genes (Martin et al. 2003).

The NBS-LRR regions of the *Rpi-bt* locus were sequenced in six contigs totaling 52 kbp covering a region of approximately 75 kbp. This region contains a cluster (Meyers et al. 2005) of six complete NBS-LRR genes (*Rpi-bt1-6*) as well as three truncated genes (Fig. 2). As shown in Fig. 2, four of the six complete genes were found to be pseudogenes, with coding sequences interrupted by either frame shift mutations or premature stop codons. These data suggested that late blight resistance at this locus was associated with *Rpi-bt2* and/or *Rpi-bt3* expression.

Identification of *Rpi-bt* Late Blight Resistance

Initial experiments focused on the two *Rpi-bt* genes with complete NBS-LRR coding sequences. To determine the efficacy of either *Rpi-bt2* or *Rpi-bt3* in conferring late blight resistance, these genes plus at least 3 kbp of 5' and 3' flanking sequence were mobilized into susceptible potatoes by *Agrobacterium*-mediated transformation. Transgenic potato plants containing either the *Rpi-bt2* or *Rpi-bt3* transgenic construct were screened for resistance to late blight by detached leaf assay (Trognitz et al. 1995). Both *Rpi-bt2* and *Rpi-bt3* genes failed to confer resistance to *P. infestans* in the detached leaf assay (data not shown). To examine the efficacy of *Rpi-bt2* and *Rpi-bt3* to confer resistance to late blight in vivo, transgenic potato plants

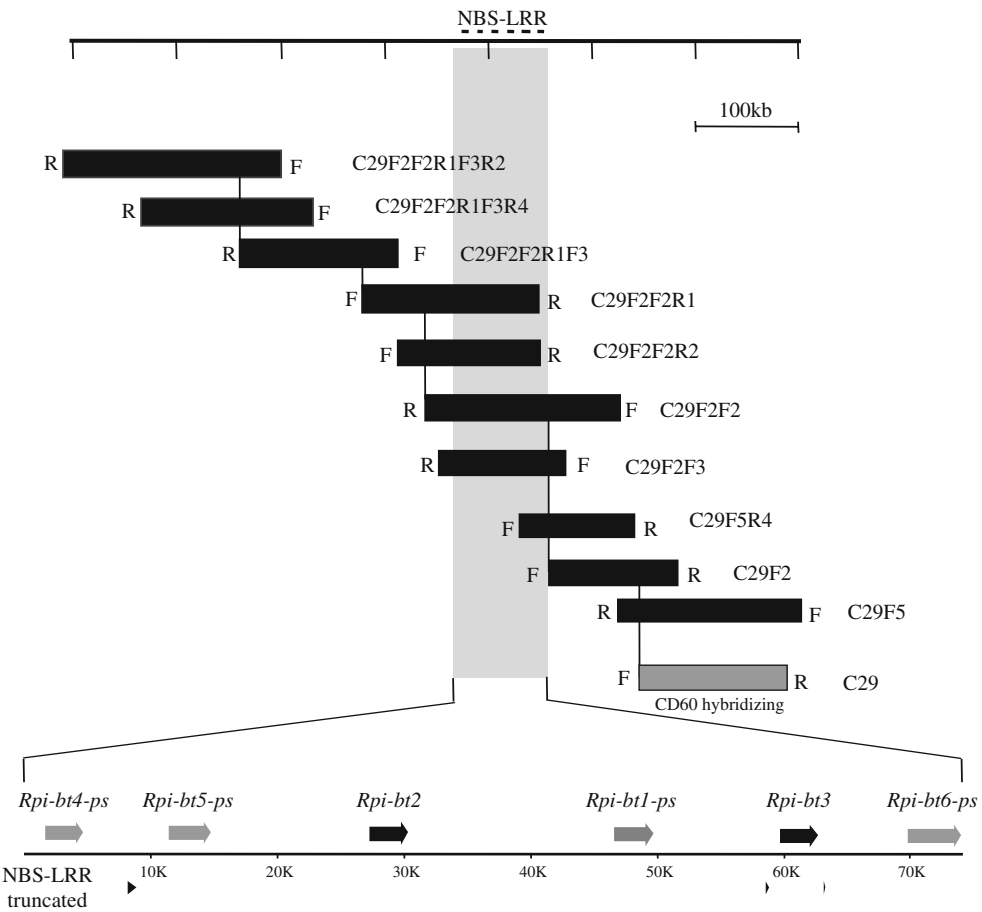

Fig. 2 Physical map of the *Rpi-bt* NBS-LRR resistance locus. The assembly of the 75 kbp contig on *S. bulbocastanum* chromosome 8 was anchored by a BAC clone hybridizing to the RFLP marker CD60. Primer pairs from BAC end-sequence used to identify overlapping BACS are indicated by the vertical lines. BAC sequence analysis revealed the *Rpi-bt* locus on BAC C29F2F3. Subcloning and sequencing of this BAC revealed six complete and three truncated NBS-LRR coding sequences. Two of the six complete genes, *Rpi-bt2* and *Rpi-bt3*, were found to encode uninterrupted open reading frames (*dark arrows*). The remaining four complete genes labeled as pseudogenes (*light arrows*) are interrupted by frame shift mutations (*Rpi-bt1-ps* and *Rpi-bt4-ps*) or premature stop codons (*Rpi-bt5-ps* and *Rpi-bt6-ps*)

were grown in the glass house and scored for disease after inoculation with *P. infestans* US-8 genotype (data not shown). None of the transgenic lines exhibited levels of resistance different than controls in two separate time course experiments.

The similarity of the NBS-LRR proteins of the *Rpi-bt* locus (Fig. 2) to the proteins encoded by known disease resistance genes is significant. In addition, the PCR screening with the BAC-end sequence primers (C29F2, and C29F5) from the *Rpi-bt* locus indicated linkage to resistance in the segregating population. Since transgenic lines containing the gDNA for *Rpi-bt2* and *Rpi-bt3* did not confer resistance in the detached leaf assays, expression profiles for these two genes were examined in *S. bulbocastanum*. Specific primers were prepared to the *Rpi-bt2* and *Rpi-bt3* genes and RACE-PCR was employed to amplify potential mRNAs from polyA$^+$ RNA isolated from *P. infestans*-infected *S. bulbocastanum* leaves. Messenger RNA products corresponding to *Rpi-bt2* and *Rpi-bt3* were not amplified. Instead transcripts similar to *Rpi-bt2* and *Rpi-bt1* were isolated. It was therefore possible that one or more of the four pseudogenes present in the *Rpi-bt* locus represented an inactive allele of a gene active on the other chromosome of this diploid species (Song et al. 2003).

A BLAST search of the GenBank non-redundant database (Altschul et al. 1990) using the deduced amino acid sequence of *Rpi-bt1-ps* corrected for the insertional frame shift returned resistance gene analog (RGA) proteins at highest identity (75%). Since the *Rpi-bt2* and *Rpi-bt3* genes were not identified in the RACE-PCR of infected *S. bulbocastanum* leaves, this suggested that the *Rpi-bt* locus might be heterozygous in *S. bulbocastanum*, with one allele active and others such as *Rpi-bt1-ps* interrupted by frame shift or stop mutations (Helgeson et al. 1988). A messenger RNA transcript was amplified with *Rpi-bt1-ps* specific primers from a late blight resistant BC3 line that contained a complete coding sequence lacking the *Rpi-bt1-ps* frame shift and was designated *Rpi-bt1* . The *Rpi-bt1* deduced amino acid sequence is 94% identical to the predicted amino acid sequence of *Rpi-bt1-ps* corrected for the frame

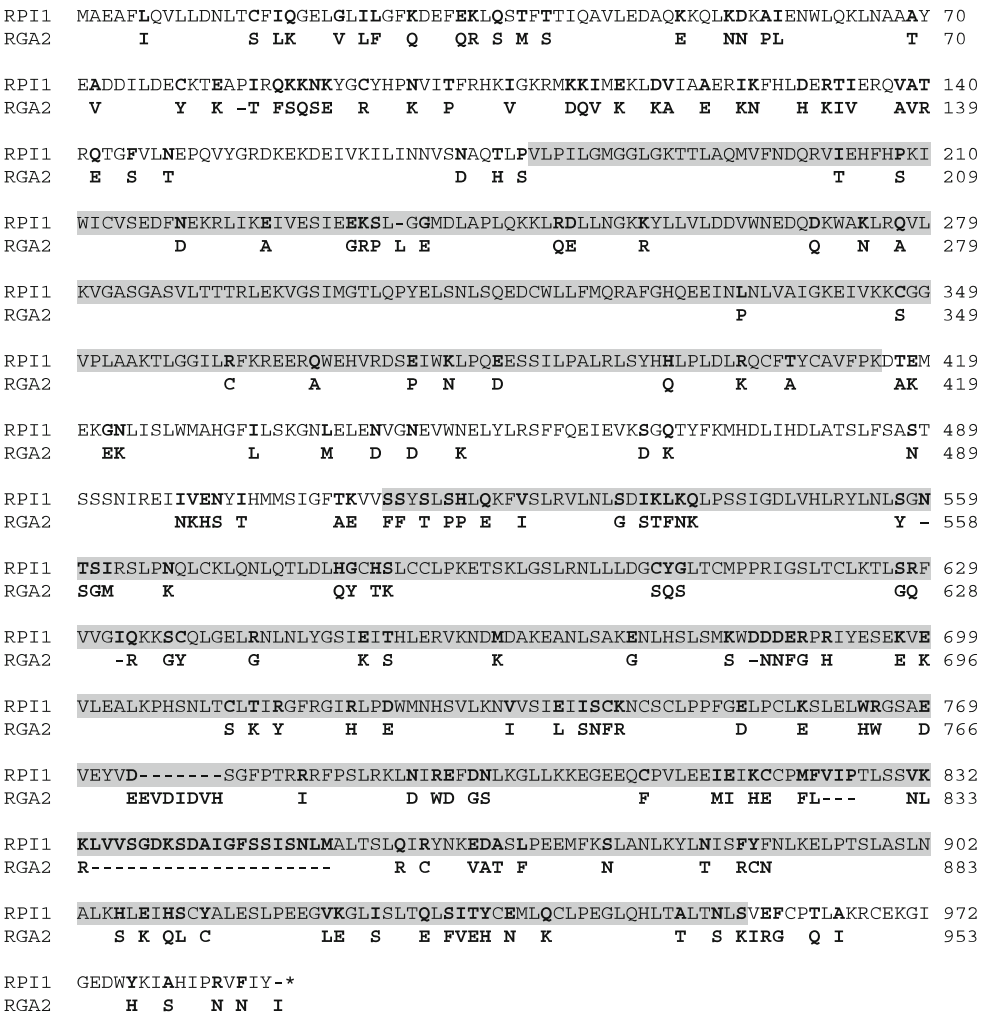

Fig. 3 Alignment of the deduced amino acid sequences of *Rpi-bt1 (*GenBank Accession FJ188415) with RGA2 (GenBank Accession Q7XA41). Only RGA2 residues that differ from RPI1 are shown, relative gaps are indicated by dashes. Shaded regions indicate NBS (*Rpi-bt1* residues 178-415) and LRR (*Rpi-bt1* residues 515-957) domains

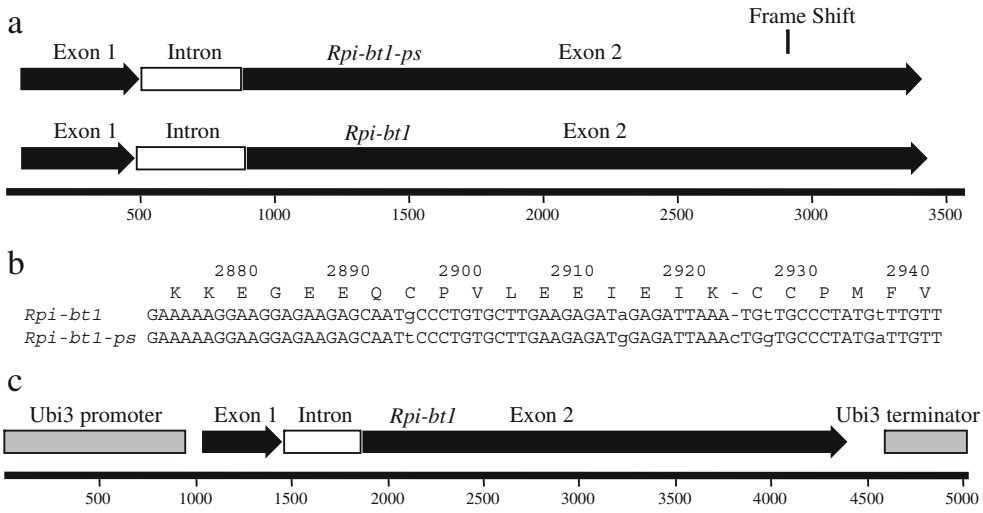

Fig. 4 Structures of *Rpi-bt1-ps* and *Rpi-bt1* genes and the chimeric transgene. **a** Comparison of structures of the *Rpi-bt1-ps* pseudogene from BAC C29F2F3 and the transcriptionally active *Rpi-bt1* genomic copy isolated by PCR. The position of the frame shift mutation in the pseudogene is indicated. **b** Alignment of *Rpi-bt1* genomic sequences in the region of the frame shift mutation in the *Rpi-bt1-ps* pseudogene. Position numbers and translation derived from the active *Rpi-bt1* gene. **c** Structure of the genomic DNA *Rpi-bt1* chimeric transgene employed in complementation analysis

shift. PCR amplification of *Rpi-bt1* from genomic DNA isolated from a late blight-resistant BC3 line generated an amplicon encoding an ORF 100% identical to the *Rpi-bt1* cDNA with a 412 bp intron. Amplification of *Rpi-bt1-ps* and *Rpi-bt2* was only successful from the BAC library and *S. bulbocastanum* gDNA and was not present in the BC3 population (data not shown) indicating the *Rpi-bt* allele represented in the BAC library was not the allele present in the BC3 population segregating for resistance.

The source of the *Rpi-bt1* gene was confirmed by PCR analysis and restriction enzyme digestion. The primers used to isolate *Rpi-bt1* cDNA and gDNA were designed from the *Rpi-bt1-ps* of the *S. bulbocastanum* BAC sequence. The *Rpi-bt1* genomic copy was isolated from the BC3 line and was not present in the BAC library. The *S. bulbocastanum Rpi-bt1* cDNA is 100% identical with the BC3 gDNA (minus intron). The gDNA of *Rpi-bt1* is 95.6 % identical to *Rpi-bt1-ps* from the *S. bulbocastanum* BAC, which is within the variation expected for alleles and highly suggestive that *Rpi-bt1* is the active allele of the *Rpi-bt* locus isolated from the *S. bulbocastanum* BAC library. Both the BAC and BC3 gDNA sequences encode a unique EcoRI restriction site in the intron. Amplification of gDNA from *S bulbocastanum* and the *Rpi-bt1* gDNA produced the predicted 554 bp PCR fragment which was subsequently cleaved by restriction endonuclease EcoRI into two fragments of 373 and 181 bp. Fragments amplified from the BC1 and BC2 parents 'Katahdin' and 'Atlantic' were not cut by EcoRI (result not shown). The *Rpi-bt* locus was also absent from the susceptible BC3 parent A86102-6 confirming *S. bulbocastanum* as the source of *Rpi-bt1*.

The predicted amino acid sequence from the *Rpi-bt1* gDNA is 91% identical to the predicted amino acid sequence of *Rpi-bt1-ps* (insertion removed). Subsequent to the isolation of *Rpi-bt1*, Song et al. (2003) reported isolation of the RB locus from *S. bulbocastanum*. A current BLAST search of the GenBank non-redundant database (Altschul et al. 1990) using the deduced amino acid sequence of *Rpi-bt1* returns the proteins RGA1, RGA2 and RGA3 from the *RB* resistance locus (Song et al. 2003; van der Vossen et al. 2003) as the most significant alignments at 76-78% identity. The deduced amino acid sequence for RPI-BT1 predicted from the gDNA of *Rpi-bt1* is most similar to the deduced amino acid sequence for RGA2 (the functional *RB* gene that confers late blight resistance) at 78% identity and 86% positive homology. The amino acid sequence alignment of RPI-BT1 and RGA2 is shown in Fig. 3.

In order to express *Rpi-bt1* in transgenic plants a chimeric transgene was constructed. Transcription of the *Rpi-bt1* gene is directed from the potato *Ubi3* promoter, which will result in constitutive moderate-level expression (Garbarino and Belknap 1994a; Garbarino and Belknap 1994b). The *Ubi3* polyadenylation signal was fused to the 3' end of the

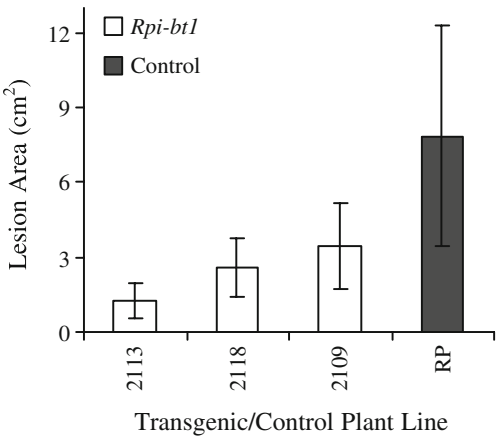

Fig. 5 Analysis of the *Rpi-bt1* resistance gene in detached leaf assays. Lesion area in transgenic potato lines expressing the chimeric *Rpi-bt1* transgene compared to the control cv. Red Pontiac (RP). Data represent the mean and standard deviation of 7 to 9 leaves (average 8.5) per plant line assessed 4 days after inoculation

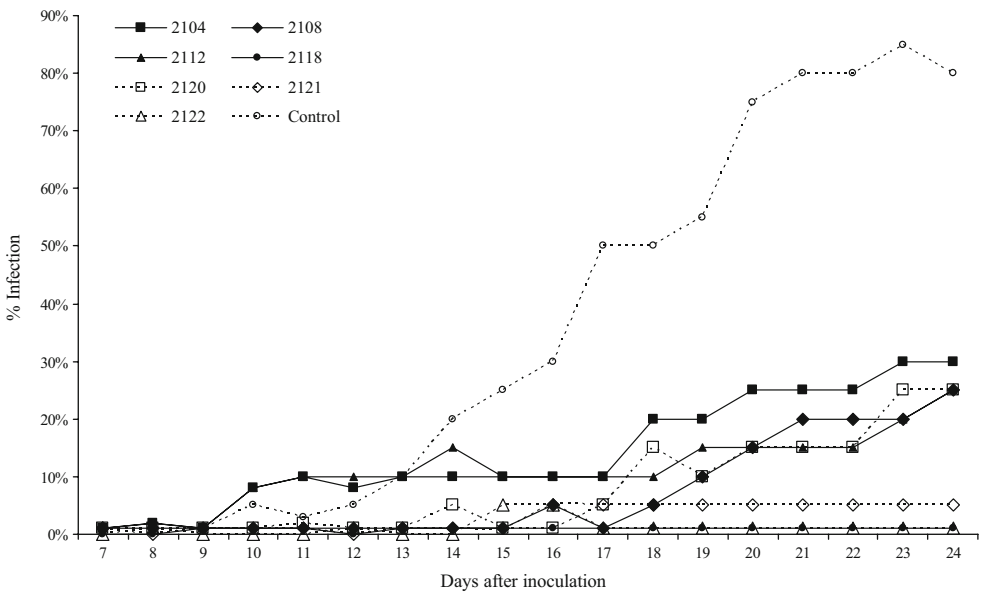

Fig. 6 Complementation analysis of the *Rpi-bt1* gene in whole plant assays. Time course and extent of infection in selected glass-house grown *Rpi-bt1 transgenic* and control cv. Atlantic potatoes. Data represents a single pot per plant line from a representative inoculation and time course

sequence (Fig. 4). The transgene was mobilized into the binary transformation vector pBINPLUS/ARS (Belknap et al. 2008; McCue et al. 2006). This binary vector construct was employed to introduce the transgene into the susceptible potato variety Atlantic. Transgenic potato plants were screened for resistance to late blight by detached leaf assay (Trognitz et al. 1995). Lesion size in the transgenic lines was reduced compared to the Red Pontiac control (Fig. 5). To examine the efficacy of *Rpi-bt1* to confer resistance to late blight in vivo, transgenic potato plants were grown in the glass house and scored for disease after inoculation with *P. infestans* US-8 genotype (Fig. 6). In contrast to the experiments with *Rpi-bt2* and *Rpi-bt3*, six of the transgenic lines had intermediate levels of resistance, and two of the transgenic lines (2,118 and 2,122) exhibited no infection 24 days after inoculation.

As shown in Figs. 5 and 6, the *Rpi-bt1* transgene constructed from the genomic cDNA conferred resistance to *P. infestans* in transgenic potatoes. While the *Rpi-bt2* and *Rpi-bt3* genes do not, individually, confer a resistant phenotype, this does not preclude a role for these genes or additional alleles in enhancing *Rpi-bt1*-mediated resistance originating from this locus.

Conclusion

Infection by *P. infestans* remains a threat to potato growers worldwide. Identifying sources of horizontal and vertical resistance to the various races of *P. infestans* remains a challenge as the genetics of the oomycete will evolve in parallel with the identification and deployment of resistance genes and strategies. Establishing durable resistance to the commonly encountered races of *P. infestans* will have an important but potentially limited life. The wild potato relative *S. bulbocastanum* is a good source of strong horizontal resistance to infection by a wide number of *P. infestans* races. Two separate loci *RB/Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003)and *Rpi-blb2* (van der Vossen et al. 2005) have now been described, each containing multiple RGAs. Additional functional genes from these loci may also confer resistance to other pathogens and diseases in the future. However, neither introgressed loci nor single genes have proven to confer the level of resistance found in the wild parent. Similarly the *Rpi-bt1* gene described here is not sufficient to confer the level of field resistance observed in the wild parent. This may be due in part to its expression via the constitutive *Ubi3* promoter. We have subsequently isolated a native promoter fragment for *Rpi-bt1* which is available for future studies. It is also possible that genes from both loci are required for the wild type levels of resistance seen in *S. bulbocastanum*. As a first step experiments are underway to combine the *RB* and *Rpi-bt1* genes in a single line using a transgenic approach. The Primers used to identify the *S. bulbocastanum* as the source of the *Rpi-bt1* gene may be useful as markers for breeders in combination with the markers for *RB* (Colton et al. 2006) to combine both of these loci. Hopefully the *Rpi-bt1* gene in concert with additional R genes from *S. bulbocastanum* or other potato relatives a strong durable resistance to infection by *P. infestans* will be achieved.

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