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Late Blight Resistance of *RB* Transgenic Potato Lines

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Abstract. Late blight of potato (*Solanum tuberosum* L.), incited by *Phytophthora infestans* (Mont.) de Bary, is a devastating disease affecting tuber yield and storage. Recent work has isolated a resistance gene, *RB*, from the wild species *Solanum bulbocastanum* Dun. Earlier work in Toluca, Mexico, observed significant levels of field resistance under intense disease pressure in a somatic hybrid containing *RB*. In this study, five transgenic *RB* lines were recovered from the late blight susceptible line MSE149-5Y, from the Michigan State University (MSU) potato breeding program. Transgenic lines were molecularly characterized for the *RB* transgene, *RB* transcript, and insertion number of the kanamycin resistance gene *NPTII*. Transgenic lines and the parent line were evaluated for resistance in field and laboratory tests. Molecular characterization alone did not predict which lines were resistant. Three of the *RB* transformed MSE149-5Y lines showed increased resistance under field conditions at MSU and increased resistance in detached leaf evaluations using multiple isolates individually (US-1, US-1.7, US-8, US-10, and US-14). Transfer of *RB* into late blight susceptible and resistant lines could provide increased protection to potato late blight. The use of the *RB* gene for transformation in this way creates a partially cisgenic event in potato because the gene's native promoter and terminator are used. This type of transformation provides a chance to generate greater public acceptance of engineered approaches to trait introgression in food crops.

Late blight of potato incited by *Phytophthora infestans* is responsible for significant annual losses in North America (Guenthner et al., 1999) and worldwide (Hijmans, 2003). The effect of climate change on potato production (Hijmans, 2003) and potato late blight (Baker et al., 2005) may result in reduced yield and increased risk of development of epidemics of potato late blight. Frequent fungicide spray intervals and rates currently used by growers to control late blight are expensive. Host resistance is an alternative control measure that is more economically and environmentally sustainable.

In the early 1900s, potato breeders successfully introgressed resistance from the Mexican wild species *Solanum demissum* Lindl. into cultivated potato (Muller and Black, 1952). However, major gene resistance from *S. demissum* was quickly overcome by *P. infestans* (Wastie, 1991). A total of 11 major dominant resistance genes (*R* genes) were identified from *S. demissum* (Malcolmson, 1969; Malcolmson and Black, 1966; Muller and Black, 1952). Most *S. demissum R* genes have been located to potato chromosomes and *R1* and *R3a* have already been sequenced (Ballvora et al., 2002; Bradshaw et al., 2006; Huang et al., 2005). Although these genes have been defeated by *P. infestans*, there is some evidence that they may be useful when combined with other sources of resistance (Stewart et al., 2003).

As races of *P. infestans* overcame resistance from *S. demissum* researchers have turned to other *Solanum* L. species for resistance genes. A multitude of genes have been mapped, and several have been cloned and sequenced. The resistance gene $R_{Pi\text{-}ber}$, from *Solanum berthaultii* Hawkes, was mapped to chromosome X (Ewing et al., 2000; Rauscher et al., 2006). *Rpi-moc1*, identified in *Solanum mochiquense* Ochoa, mapped to chromosome IX (Smilde et al., 2005). *Rpi-phu1* was identified from *Solanum phureja* Juz. et Buk. and mapped to chromosome IX (Śliwka et al., 2006). *Rpi1* from *Solanum pinnatisectum* Dun. was mapped to chromosome VII (Kuhl et al., 2001). A major QTL from *Solanum microdontum* Bitt. (Bisognin et al., 2005) was recently mapped to chromosome IV (D.S. Douches, unpublished data). *Solanum bulbocastanum* has yielded several resistance genes. Two alleles from a single locus on chromosome VIII were identified and cloned, *RB* (Song et al., 2003) and *Rpi-blb1* (van der Vossen et al., 2003). *Rpi-blb2* was localized to chromosome VI and subsequently cloned (van der Vossen et al., 2005). Another gene, *Rpi-blb3*, has been mapped to chromosome IV (Park et al., 2005).

The increasing pool of genes conveying resistance to late blight of potato raises the question of how to incorporate such resistance into cultivated lines. The cultivated potato, *S. tuberosum*, is tetraploid (4*x*) with an endosperm balance number (EBN) of 4, resulting in crossing barriers with wild *Solanum* species that differ in ploidy or EBN (Hanneman, 1999). Although classical methodologies have been used to overcome such barriers (Hermsen and Ramanna, 1973), the desire to incorporate more than one source of resistance presents increased challenges for the potato breeder. Plant transformation offers an efficient method to transfer genes from divergent organisms directly into the plant genome (Sharma et al., 2005). Transformation also allows both the quantity, through gene pyramiding, and timing of resistance genes to be controlled on a relatively short time scale.

Resistance genes are potentially useful control measures for potato late blight. Both *RB* and *Rpi-blb1* have broad-spectrum resistance and conferred resistance to a range of isolates of *P.*

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infestans carrying multiple virulence factors (Song et al., 2003; van der Vossen et al., 2003). *RB* has also been shown to maintain resistance in Toluca, Mexico under intense disease pressure (Helgeson et al., 1998). However, successful insertion of a transgene into the genome does not guarantee a positive phenotype. van der Vossen et al. (2003) reported that 15 of 18 *Rpi-blb1* transformed potato lines were resistant and seven of nine tomato (*Solanum lycopersicum* L.) transformants showed resistance. Song et al. (2003) reported that of 14 *RB* transformed lines, 5 were highly resistant and 9 were moderately resistant, having <10% and 11% to 25% infection, respectively, in comparison with non-transformed lines. Successful transformation requires confirmation that the gene or genes are not only intact and transcribed/translated but that the desired phenotype is also recovered.

The objectives of this study were to evaluate the recovery of effectively transformed progeny plants of the potato advanced breeding line MSE149-5Y and to determine the resistance of these transformed lines to different genotypes of *P. infestans* in detached leaf tests and the field.

Materials and Methods

Generation of transgenic lines. Michigan State University breeding line MSE149-5Y was used for all transformations reported here. MSE149-5Y is not known to have any major late blight *R* gene. Plasmid 2.4.48pCRXLTOPO (J. Jiang, personal communication) was digested with *Bam*HI, and the 8.59-kb fragment containing *RB* was isolated, including 2.5 kb upstream of the start ATG and 2.48 kb downstream of the stop codon. This fragment was ligated to pBINPLUSARS (U.S. Department of Agriculture, Albany, CA) (Garbarino and Belknap, 1994), which had been digested with *Bam*HI, and the new construct called pSPUD69 (Fig. 1). *Agrobacterium tumefaciens* (Smith & Towns.) Conn.-mediated transformations using a kanamycin marker were conducted according to Douches et al. (1998). The explants were prepared for transformation by cutting internodes of the stem from tissue culture plantlets. When callus nodules produced shoots 5–7 mm in length, the shoots were excised and placed in rooting medium (modified MS medium with the addition of kanamycin at 50 mg L^{-1} to identify successful transformants) in 25 × 150-mm culture tubes. A single shoot was removed from each callus to ensure selection from independent transformation events. Rooted transformants expressing resistance to kanamycin were maintained by micropropagation. Rooted putative transgenic plants in the MSE149-5Y background were denoted as E69 followed by the shoot number.

Molecular characterization. DNA extractions were conducted using a 2× CTAB procedure (Doyle and Doyle, 1987). Detection of transgene insertions used 30 ng of total genomic DNA at a final concentration of 1.5 mg L^{-1}. RED*Taq* DNA Polymerase (Sigma-Aldrich Co., St. Louis) was used following the manufacturer's directions. *RB* primers specific for the resistant allele (Colton et al., 2006) amplified a 213-bp fragment (forward, 5′ CACGAGTGCCCTTTTCTGAC 3′ and reverse, 5′ ACAATTGAATTTTTAGACTT 3″). *NPTII* primers amplified a 267-bp fragment (forward 5′ CGCA GGTTCTCCGGCCGCTTGGGTGG 3′ and reverse, 5′ AGCA GCCAGTCCCTTCCCGCTTCA 3′). *RB* and *NPTII* primers were annealed at 43 and 60 °C, respectively. A PerkinElmer 9600 thermal cycler (Wellesley, MA) was programmed for a hot start (95 °C, 5 min) and 35 cycles of 95 °C, 30 s denaturing, 43 °C or 60 °C, 30 s annealing, and 72 °C, and 30 s extension, with a final extension of 72 °C, 5 min. Fragments were separated on 1.0% agarose gel.

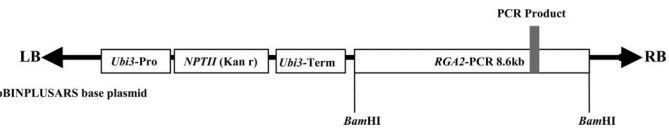

Fig. 1. T-DNA region of pSPUD69. *RGA2*-PCR indicates the 8.59-kb *Bam*HI fragment containing the *RB* gene ligated into the pBINPLUSARS base plasmid. This construct includes the *NPTII* gene for kanamycin resistance controlled by the ubiquitin (*Ubi3*) promoter (Pro) and terminator (Term). Natural promoter (2503-bp upstream) and termination sequences (2477-bp downstream) were used for *RB*. The region in the T-DNA where the *RB* primers anneal is designated by "PCR Product."

RNA extractions were conducted using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA). One microgram of RNA was treated with deoxyribonuclease I (Invitrogen Corp., Carlsbad, CA) and inactivated according to the manufacturer's guidelines. cDNA was generated from total RNA using M-MLV Reverse Transcriptase (Invitrogen Corp.) according to the manufacturer's guidelines. Polymerase chain reaction (PCR) was conducted with RED*Taq* DNA Polymerase (Sigma-Aldrich Co.) according to the manufacturer's guidelines. *RB* and *NPTII* primers were the same as above. Positive control was *EF1α* (forward, 5′ GGTGGTTTTGAAGCTGG TATCTCC 3′ and reverse, 5′ CCAGTAGGGCCAAAGGTCA CA 3′), 50 °C annealing temperature. Fragments were separated on 1.0% agarose gel.

Southern hybridizations. Twenty micrograms of endo-restriction-digested (*Bam*HI, *Eco*RI, and *Hin*dIII) DNA per sample was transferred to Amersham Hybond-N^+ membrane (GE Healthcare, Piscataway, NJ) using a standard alkaline lysis procedure (Kennard et al., 1994). Isolated fragments of *RB* and *NPTII* were labeled with [α-^{32}P]dCTP with the Random Primers DNA Labeling system (Invitrogen Corp.), and un-incorporated nucleotides were removed with Bio-Spin 30 Tris columns (Bio-Rad, Hercules, CA). Hybridizations were conducted as described in Kuhl et al. (2001). Hybridization with *RB* used a 1562-bp fragment, bp 1794 through bp 3356 from ATG, including the 213-bp region used to distinguish resistant and susceptible alleles. Highly stringent final wash conditions were used 0.1× SSC and 0.1% SDS for a total of 52 min at 60 °C. Hybridization with *NPTII* used a 700-bp fragment. The final wash condition included 2× SSC, 0.1% SDS for 30 min at 50 °C followed by 0.1× SSC, 0.1% SDS for 20 min at 60 °C.

Detached leaf tests. Isolates of different genotypes of *P. infestans* used in detached leaf tests are described in Table 1. Isolates were transferred through healthy, detached leaflets of a susceptible cultivar (Red Craig's Royal, not known to contain any R-genes) within 1 year of the conducted tests. Sporangial/zoospore suspensions were derived from each isolate using three to five 13-d-old cultures grown on rye A agar (Caten and Jinks, 1968). Sporangia were released from the surface of the plate by immersion in sterile distilled water (SDW), followed by scraping with a sterile glass rod. Zoospore release was stimulated by incubation for 2 h at 4 °C, followed by warming at 18 °C for 30 min. Suspensions were subsequently examined using a binocular microscope to confirm the release of zoospores, calibrated by counting with a hemocytometer and

Table 1. *Phytophthora infestans* isolates independently used in potato detached leaf evaluations at 2.0×10^4 sporangia/mL sprayed on the abaxial leaf surface and incubated at 17 °C for 12 d.

Isolate	Genotype[z]	Mating type	*R*-gene phenotype[y]
Pi95-3[x]	US-1	A1	5
Pi95-2	US-1.7	A2	1, 9
Pi02-007	US-8	A2	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
SR83-84	US-10	A2	1, 2, 4, 5, 10
Pi99-2[x]	US-14	A2	1, 5

[z]Genotypes as designated by Goodwin et al. (1995) and Young et al. (2004).
[y]Virulence phenotype as defined by Muller and Black (1952).
[x]Less than 2.0×10^4 sporangia/mL for one or both detached leaf inoculations.

adjusted to about 2.0×10^4 sporangia/mL using SDW (Kuhl et al., 2001).

Inoculations were conducted essentially according to Kuhl et al. (2001) on 12 Dec. 2004 and replicated 3 Jan. 2005. Five plants per line of greenhouse-grown plants were used. Five fully developed leaves were selected from the upper portion of each plant and one leaf randomly assigned per *P. infestans* isolate, so that five leaves total were used per isolate. Three layers of absorbent paper towels were placed in the bottom of 150-mm-diameter petri dishes with a layer of plastic mesh on top, and 38 mL of SDW was added to the towels in each plate. Detached leaves were placed abaxial side up on the plastic mesh and then sprayed using a hand-held sprayer, once with SDW and once with *P. infestans* inoculum (0.7-mL spray volume per plate per spray). Petri dishes were wrapped with parafilm and placed into dark incubators at 17 °C with 12-h light cycle. For all experiments incubator shelves and positions were randomized. Pi95-3 (US-1) and Pi99-2 (US-14) did not produce sufficient sporangia although $\approx 1.0 \times 10^3$ sporangia/mL were used at both inoculations for Pi95-3 and $\approx 2.0 \times 10^4$ and $\approx 1.4 \times 10^3$ sporangia/mL were used for Pi99-2 at inoculations 1 and 2, respectively. Leaves were scored at 6, 9, and 12 d after inoculation (DAI) using disease severity indices (DSI) based on percentage of leaf area affected: 0 = no symptoms, 1 = 0% to 5%, 3 = 6% to 25%, 5 = 26% to 50%, 7 = 51% to 75%, 9 = 76% to 100% (Kuhl et al., 2001). Scores were converted to mean percentage values by using the midpoint value in each interval (Haynes et al., 1998). Percentages were used to calculate area under the disease progress curve (AUDPC) according to Shaner and Finney (1977) and analysis of variance conducted using XLSTAT (version 2006.5; Addinsoft, New York, NY).

Field evaluations. Inoculated fields trials were conducted in 2004 and 2005 at the Michigan Agricultural Experimental Station Muck Soils Research Farm, Laingsburg, MI. Plots were irrigated as necessary to maintain canopy and soil moisture conditions conducive for development of foliar late blight (Kirk et al., 2005) with turbine rotary garden sprinklers (Gilmour Group, Somerset, PA) at 1.05 $L \cdot ha^{-1}$ of water per hour and managed under standard potato agronomic practices.

In 2004, five tissue-culture-derived plants per plot were transplanted to the field and each line replicated twice. Inoculation in 2004 used a mixture of isolates later used in detached leaf test and listed in Table 1, except for SR83-84 (US-10). In 2005, 10 tissue-culture-derived plants per plot were transplanted to the field and replicated 3 times. The most promising resistance lines were retested in 2005, thereby dropping E69.03 and E69.04. All the isolates listed in Table 1 and isolates Pi96-2 (US-1.7), Pi95-7, Pi02-003, Pi02-006 (US-8), Pi96-1 (US-11), and Pi94-2, Pi98-1, and Pi00-001 (US-14) were used to inoculate the field plots in 2005. Sporangia were counted with a hemacytometer and the concentration was adjusted to $\approx 2.0 \times 10^4$ sporangia/mL using SDW. The sporangial solution was chilled to 4 °C for 8 h to promote release of zoosporangia from the sporangia. All plots were inoculated simultaneously in both years using an overhead sprinkler irrigation system. The zoospore suspension of mixed isolates of *P. infestans* was injected into the irrigation water feed pipeline under 0.5 $kg \cdot cm^{-2}$ of CO_2 pressure and applied at a rate of ≈ 150 $mL \cdot m^{-2}$ of inoculum solution of trial area to give an inoculation rate of about 2.4×10^6 zoosporangia/m^2 (2.4×10^{10} zoospores$\cdot ha^{-1}$). This amount and rate of inoculum applied were estimated from prior calibration of the irrigation system and were intended to expose all potato foliage to inoculum of *P. infestans*.

As soon as late blight symptoms were detected (about 7 DAI), each plant within each plot was visually rated at 3- to 5-d intervals for percent leaf and stem (foliar) area with late blight lesions. The mean percent blighted foliar area per treatment was calculated. Evaluations continued until plots of susceptible cultivars reached 100% foliar area diseased. DAI to the time until 100% foliar area in susceptible cultivars diseased was reached was used as a key reference point for calculation of relative area under the disease progress curve [RAUDPC (Kirk et al., 2001)]. Analysis of variance was calculated using JMP (version 5.0.1; SAS Institute Inc., Cary, NC).

Results

Molecular evaluations. PCR tests on total genomic DNA revealed five transformants of MSE149-5Y transformed for both *RB* and *NPTII* (Fig. 2). In addition to the two lines in Fig. 2, E69.02 and E69.10, >150 other lines tested positive for *NPTII* but failed to amplify the fragment from *RB* (data not shown). As a consequence, screening of newly transformed lines required testing for both *NPTII* and *RB*.

In an effort to determine copy number of the transformed plants, a Southern blot was hybridized with the *RB* fragment. The *RB* fragment used includes sequence before the leucine-rich repeat (LRR) region and 1113 bp of LRR, including the 213-bp region used to distinguish *RB* from other alleles. In anticipation that the labeled fragment would hybridize to numerous genes in *S. tuberosum*, a highly stringent final wash was used. Despite this effort, the *RB* fragment hybridized to many regions within the potato genome (data not shown). No bands could be identified as specific to the *RB* transgene. Due to the nature of the construct used (Fig. 1), *NPTII* could be used to approximate *RB* copy number.

A 700-bp fragment from *NPTII* was hybridized to the five *RB* transformed lines (Fig. 3). Four of the lines showed a single labeled fragment—E69.01, E69.03, E69.04, and E69.06—suggesting the transgene inserted into a single site in the genome. Lane 4, E69.05, contains two *NPTII* labeled fragments, suggesting two separate insertion events.

Reverse-transcribed PCR (RT-PCR) was used to evaluate for transcription of the *RB* and *NPTII* transgenes. Lines E69.01–E69.06 detected *NPTII* transcript, while *RB* transcript was detected in lines E69.03, E69.04, E69.05, and E69.06 (Fig. 4). Notably, *RB* transcript was not detected in line E69.01, even though the *RB* fragment amplified from total genomic DNA

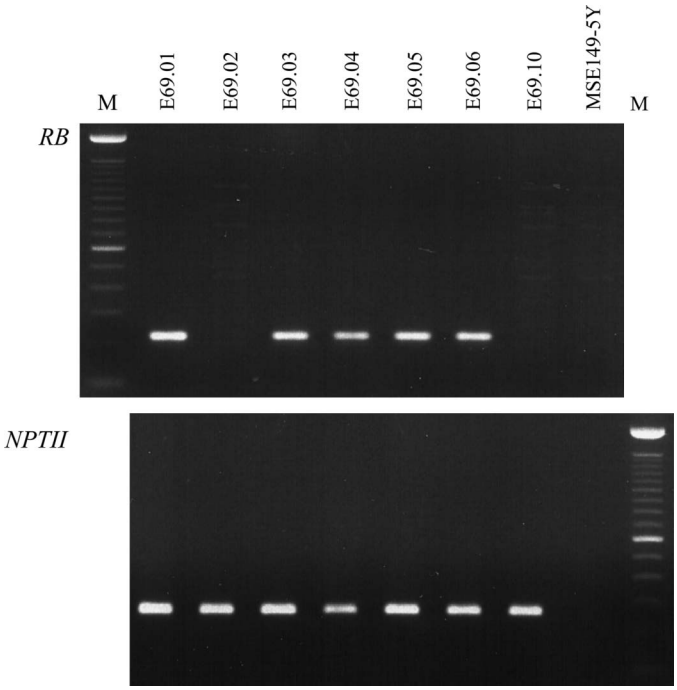

Fig. 2. PCR amplification of *RB* and *NPTII* fragments from total potato DNA. The expected *RB* fragment is 213 bp. Lanes E69.01, E69.02, E69.03, E69.04, E69.05, E69.06, and E69.10 are putative transformants; lane MSE149-5Y, untransformed control; lane M, 100-bp DNA ladder.

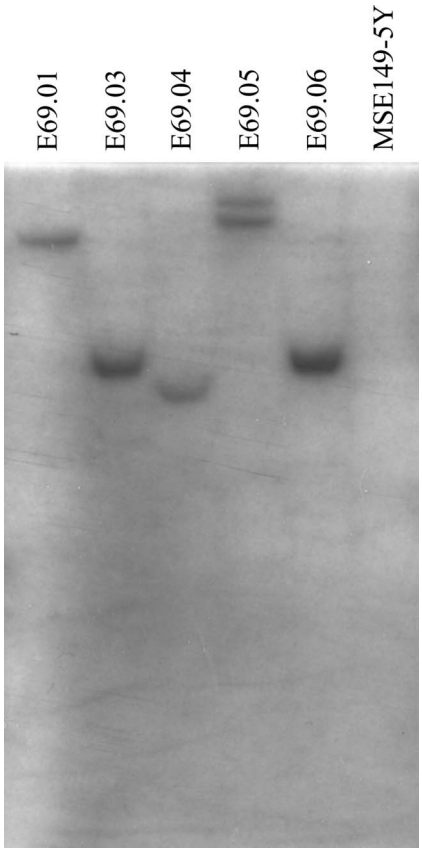

Fig. 3. Southern hybridization of *Bam*HI-digested total potato DNA with *NPTII* 700-bp fragment, 5-d exposure. Lanes E69.01, E69.03, E69.04, E69.05, and E69.06 are putative transformants; lane MSE149-5Y, untransformed control.

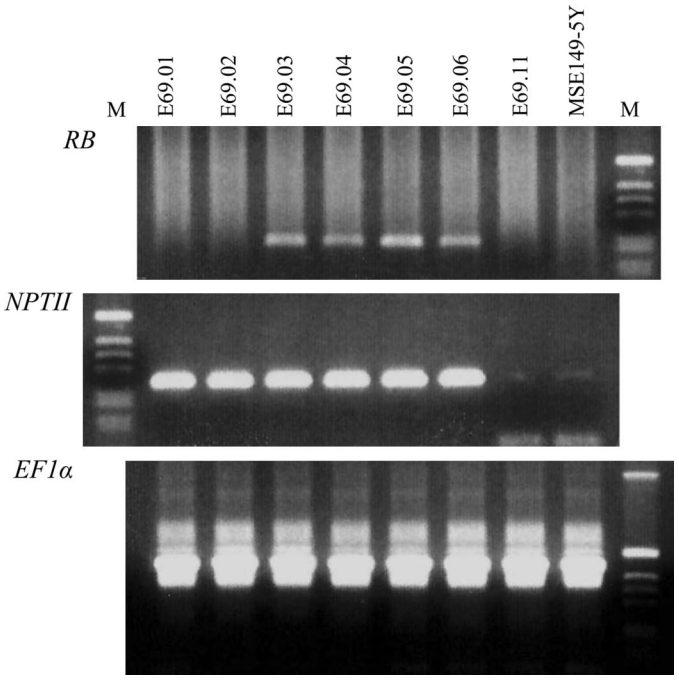

Fig. 4. Reverse-transcribed PCR of *RB*, *NPTII*, and *EF1α*. Expected fragments of *RB*, *NPTII*, and *EF1α* are 213, 267, and 400 bp, respectively. Lanes E69.01, E69.02, E69.03, E69.04, E69.05, E69.06, and E69.11 are putative potato transformants; lane MSE149-5Y, untransformed potato control; lane M, 1-kb DNA ladder.

(Fig. 2). The absence of *RB* transcript is expected for E69.02 and E69.11, both of which lack the amplified *RB* fragment.

Detached leaf tests. Detached leaflet tests indicated that there were no significant differences in AUDPC among lines of MSE149-5Y transformed with the *RB* gene inoculated with *P. infestans* isolates Pi95-3 (US-1), Pi95-2 (US-1.7), Pi02-007 (US-8), or Pi99-2 (US-14) (Table 2). With isolate SR83-84 (US-10), lines E69.03, E69.05, and E69.06 had significantly lower AUDPC values (31–35) than lines with AUDPC values from 154 to 190 (Table 2).

Field data. Foliar potato late blight developed steadily after inoculation in 2004, and the RAUDPC value in the untransformed line MSE149-5Y was 47.7 (41 DAI; Table 3). RAUDPC values of lines E69.03, E69.05, and E69.06 in 2004 ranged from 0.7 to 6.3 and were significantly lower ($P = 0.05$) values than the untransformed MSE149-5Y. Line E69.04 had a significantly greater RAUDPC value (27.8) than the three most resistant lines but significantly lower than the untransformed line (Table 3). In 2004, line E69.01 had a significantly greater RAUDPC value (38.8) than E69.04 (27.8) but significantly less than MSE149-5Y (47.7). In 2005, the epidemic progressed more slowly, and by 45 DAI the RAUDPC value in the untransformed line MSE149-5Y was 19.8 (Table 3). The RAUDPC value for E69.01 (19.5) was not significantly different from the value for MSE149-5Y (19.8). Lines E69.05 and E69.06 in 2004 had RAUDPC values from 1.8 to 4.5 and were significantly lower ($P = 0.05$) values than the untransformed MSE149-5Y.

Discussion

Both field and detached leaf tests detected increased levels of late blight resistance in three transformed lines, E69.03,

Table 2. Effect of transformation of potato line MSE149-5Y with *RB* on susceptibility to *Phytophthora infestans* in detached leaf tests scored at 6, 9, and 12 DAI.

	Foliar potato late blight (AUDPC)[z] on lines of MSE149-5Y transformed with *RB* gene						
Isolate	MSE149-5Y	E69.01	E69.02	E69.03	E69.04	E69.05	E69.06
Pi95-3[y]	0 a[x]	168 a	9 a	24 a	32 a	161 a	103 a
Pi95-2	22 a	59 a	26 a	9 a	49 a	122 a	33 a
Pi02-007	374 a	395 a	405 a	406 a	457 a	513 a	341 a
SR83-84	154 a	190 a	184 a	31 b	162 a	35 b	31 b
Pi99-2[w]	300 a	265 a	264 a	110 a	290 a	148 a	209 a

[z]Area under the disease progress curve derived from measuring potato late blight lesion area from inoculation to 12 DAI.
[y]Inoculated with $\approx 1.0 \times 10^3$ sporangia/mL for both inoculation dates.
[x]Values followed by the same letter within individual rows (isolates) are not significantly different at $P \leq 0.05$ using Tukey honestly significant different (HSD) pairwise comparison.
[w]Inoculated with $\approx 2.0 \times 10^4$ and $\approx 1.4 \times 10^3$ sporangia/mL for 12 Dec. 2004 and 3 Jan. 2005, respectively.

Table 3. Effect of transformation of potato line MSE149-5Y with the *RB* gene on susceptibility to *Phytophthora infestans* in field plots at the Michigan Agricultural Experimental Station Muck Soils Research Farm at Laingsburg in 2004 and 2005.

Yr	Line	*N*	RAUDPC × 100	*RB* transgene[z]	*RB* transcript[y]
2004[x]	MSE149-5Y	2	47.7	–	–
	E69.01	2	38.8	+	–
	E69.04	2	27.8	+	+
	E69.03	2	6.3	+	+
	E69.06	2	4.2	+	+
	E69.05	2	0.7	+	+
		$LSD_{0.05}$ =	3.4		
2005[w]	MSE149-5Y	3	19.8	–	–
	E69.01	3	19.5	+	–
	E69.06	3	4.5	+	+
	E69.05	3	1.8	+	+
		$LSD_{0.05}$ =	5.4		

[z]Amplification of 213-bp *RB* fragment from total DNA extraction.
[y]RT-PCR amplification of 213-bp *RB* fragment from cDNA.
[x]Isolates (genotypes) applied: Pi95-3 (US-1), Pi95-2 (US-1.7), Pi02-007 (US-8), and Pi99-2 (US-14).
[w]Isolates (genotypes) applied: Pi95-3 (US-1), Pi95-2, Pi96-2 (US-1.7), Pi02-007, Pi95-7, Pi02-003, Pi02-006 (US-8), SR83-84 (US-10), Pi96-1 (US-11), and Pi94-2, Pi98-1, Pi99-2, and Pi00-001 (US-14).

E69.05, and E69.06. In the case of E69.01 molecular data, presence of *RB* fragment (Fig. 2) but absence of *RB* transcript (Fig. 4) accurately predicted a susceptible phenotype in both laboratory and field evaluations. However, E69.04, a susceptible phenotype that contains the *RB* DNA fragment and transcript, would not have been detected without laboratory or field phenotypic testing. Phenotype screening is needed to accurately determine which transformed lines are worth carrying forward.

Detached leaf tests accurately predicted field resistance in lines E69.03, E69.05, and E69.06. This interpretation was only good for the US-10 isolate, SR83-84. Isolates Pi95-3 and Pi99-2 both produced suboptimum sporangia concentrations resulting in highly variable phenotypes. *P. infestans* US-8 isolate Pi02-007 was particularly aggressive and successfully infected all lines evaluated in detached leaf tests. This same isolate was also applied in field inoculations, but resistance was clearly observed in lines E69.03, E69.05, and E69.06. The most likely explanation is that conditions of the detached leaf test were too stringent for this isolate and that leaves were overcome by density of inoculum. Stewart (1990) found that the best inoculum concentration to distinguish resistant and susceptible phenotypes is likely to depend on the isolate. Adjustment of the inoculum concentration may help resolve susceptible and resistant phenotypes. Both *RB* and *Rpi-blb1* are reported to contribute broad-spectrum resistance to all *P. infestans* races (Song et al., 2003; van der Vossen et al., 2003). However, Pi02-007 has been observed to be a highly aggressive isolate (Young et al., 2004), suggesting that lower concentration of inoculum may be required to detect resistant phenotypes. In this report, Pi02-007 was significantly different from the other isolates ($P \leq 0.01$) with the highest mean AUDPC, 412 ± 120. Use of multiple isolates reported here allowed accurate detection of transgenic resistance in laboratory tests and corroborated field results.

Transformation efficiency was very low in this study. The low efficiency is unexpected with MSE149-5Y, which has proven to be particularly receptive (75% regeneration rate) to transformation (D.S. Douches, unpublished data). The use of the pSPUD69 construct resulted in many *NPTII* positive/*RB* negative transgenic lines. A greater number of lines should be generated so that the true range of expression of this gene can be studied. It also would be valuable to express *RB* in other late blight resistant germplasm. However, the low recovery of *RB* positive lines has restricted transfer of *RB* to conventionally bred late blight resistant lines because the late blight resistant genotypes were not responsive to callus regeneration (D.S. Douches, unpublished data). Studies must be conducted to understand this phenomenon so that greater numbers of *RB* lines can be generated for evaluation.

Determination of *RB* copy number relied on hybridization with a *NPTII* fragment. A single band was observed for four of five *RB*-containing lines, suggesting a single insertion of *RB*. However, this does not exclude the possibility of a closely linked tandem insertion or the possibility of additional *RB* insertions with the loss of *NPTII*. This highlights a challenge for researchers using potato genes to transform potato. One alternative approach is to use real-time PCR to estimate copy number (Ingham et al., 2001) using gene- and/or allele-specific primers. An unsuccessful effort using *RB*-specific primers attempted to do just that (J.C. Kuhl, unpublished data). Failure may in part be due to the low annealing temperature of the *RB* primers (43 °C) and the challenge to identify alternative

primers. *RB*, a coiled coil–nucleotide binding site–leucine-rich repeat class resistance gene (Martin et al., 2003), is expected to be homologous with other potato resistance genes, as demonstrated by hybridization with *RB*, making allele specific primers difficult to identify.

The 213-bp *RB* fragment was amplified from genomic DNA of line E69.01, but *RB* transcript was not detected. This could be explained by a partial deletion/rearrangement of the *RB* transgene, leaving intact the 213-bp DNA region, but inactivating or truncating transcription. Another possibility is post-transcriptional silencing, as observed in other transgenic lines (Felcher et al., 2003).

As transformation technologies become more efficient greater numbers of putatively transformed lines will be generated. Molecular characterization does not guarantee a resistant phenotype. Field evaluations require propagation of the desired lines and large-scale preparation and deployment of *P. infestans* inoculum, in addition to regular field observations throughout disease development. Therefore, it may not be desirable to take all putative transformed lines to the field. As evidenced by correlation of detached leaf assays and field results, screening of transformants in the laboratory before field evaluations will contribute to more efficient use of field space.

RB may be useful creating late blight resistant phenotypes in either susceptible or resistant cultivars of potato. Cultivar × fungicide studies, as previously conducted by Kirk et al. (2005), should be conducted to determine the best way to integrate protectant fungicides and host plant resistance to achieve late blight control for commercial conditions. These lines generated can be used for further breeding to introgress *RB* into additional germplasm.

The use of the *RB* gene from *S. bulbocastanum* for transformation creates a partially cisgenic event in potato because the gene's native promoter and terminator is also used. This type of transformation creates an opportunity to generate greater public acceptance of engineered approaches to trait introgression in our food crops. Currently, the *NPTII* gene is also in the vector construct T-DNA region. Transformation methods exist to create marker-free transgenics in potato (Rommens et al., 2004). Further transformations in our program have created *RB*-transgenic lines in which *RB* is inserted independently from *NPTII*. Through crossing we may be able to identify *RB*-positive/*NPTII*-negative transgenic lines for further breeding.

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