Higher Copy Numbers of the Potato *RB* Transgene Correspond to Enhanced Transcript and Late Blight Resistance Levels

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Late blight of potato ranks among the costliest of crop diseases worldwide. Host resistance offers the best means for controlling late blight, but previously deployed single resistance genes have been short-lived in their effectiveness. The foliar blight resistance gene *RB*, previously cloned from the wild potato *Solanum bulbocastanum*, has proven effective in greenhouse tests of transgenic cultivated potato. In this study, we examined the effects of the *RB* transgene on foliar late blight resistance in transgenic cultivated potato under field production conditions. In a two-year replicated trial, the *RB* transgene, under the control of its endogenous promoter, provided effective disease resistance in various genetic backgrounds, including commercially prominent potato cultivars, without fungicides. *RB* copy numbers and transcript levels were estimated with transgene-specific assays. Disease resistance was enhanced as copy numbers and transcript levels increased. The *RB* gene, like many other disease resistance genes, is constitutively transcribed at low levels. Transgenic potato lines with an estimated 15 copies of the *RB* transgene maintain high *RB* transcript levels and were ranked among the most resistant of 57 lines tested. We conclude that even in these ultra-high copy number lines, innate RNA silencing mechanisms have not been fully activated. Our findings suggest resistance-gene transcript levels may have to surpass a threshold before triggering RNA silencing. Strategies for the deployment of *RB* are discussed in light of the current research.

Late blight disease of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicon*) results in multibillion-dollar annual yield losses and production costs worldwide (Kamoun 2001), ranking it among the world's costliest crop diseases. Given the significance of potato as a food crop (Bradeen et al. 2008), the disease is especially a problem for major potato producing countries. Genotypic diversity of *Phytophthora infestans*, the late blight pathogen, and cool, humid conditions combine to make potato foliar late blight the major limiting production factor in many regions. Chemical controls are available but add production and environmental costs. Importantly, some *P. infestans* genotypes, including pathotype US8, the predominant genotype in the United States today, are resistant to systemic fungicides (Daayf et al. 2000; Marshall-Farrar et al. 1998; McLeod et al. 2001; Mukalazi et al. 2001; Olafsson and Hermansen 2001). Thus, chemical controls are applied in a prophylactic manner and may be used even when conditions ultimately do not favor epidemics. Genetic disease resistance is a viable, environmentally sustainable alternative to fungicides, and researchers have sought durable late blight resistance for more than 100 years (Black and Gallegly 1957; Niederhauser and Mills 1953; Reddick 1930, 1939; Toxopeus 1956). Foliar late blight resistance (*R*) genes from some of the approximately 200 wild potato species have been bred into *S. tuberosum*. Most notorious is the *R1* to *R11* series of genes introgressed from *Solanum demissum*. Each gene is pathogen race-specific, functioning against a limited set of pathogen genotypes. Each also induces a macroscopic hypersensitive reaction (HR), placing extreme selective pressure on the pathogen population. Initially promising, under broad field utilization, the *R1* to *R11* series was quickly defeated as the genetics of the pathogen population shifted to circumvent detection by the plant (Toxopeus 1956; Umaerus and Umaerus 1994). *R* genes from other wild potato species might prove to be more broadly applicable for the protection of cultivated potato.

The wild potato *Solanum bulbocastanum* is native to central and southern Mexico, a region recognized as a genotypic center of diversity for *P. infestans*. Conditions in parts of Mexico offer ideal conditions for late blight epidemics. It is likely that *S. bulbocastanum* and *P. infestans* have a long coevolutionary history in these regions (Niederhauser and Mills 1953). As might be expected, *S. bulbocastanum* has devised genetic means for combating late blight. Three *S. bulbocastanum* late blight resistance genes have been described: *RB* (syn. *Rpi-blb1*; [Song et al. 2003; van der Vossen et al. 2003]), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* (Park et al. 2005). These genes lack pathogen race specificity (Song et al. 2003; van der Vossen et al. 2005) and are thus distinct in phenotypic effect from the *S. demissum* *R1* to *R11* series. Lack of race specificity associated with the *S. bulbocastanum* genes might favor long-term durability. *RB*-mediated foliar blight resistance lacks a clear, macroscopic HR (Song et al. 2003). Instead, *RB* induces delayed pathogen development and spread. Lack of a strong HR might also favor durability for *RB*. Although *S. bulbocastanum* is sexually isolated from cultivated potato (Jackson and Hanneman 1999), resistance genes can be transferred be-

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color online.

tween the species using transgenic approaches. *RB* and *Rpi-bllb2* have been cloned (Song et al. 2003; van der Vossen et al. 2003, 2005). Characterization of cultivated potato lines carrying the *RB* transgene under field conditions conducive to late blight is key to large-scale gene deployment. Similarly, understanding the effects of varying transgene copy number and transcription levels on phenotypic resistance may suggest strategies for maximizing resistance.

Song and associates (2003) demonstrated that independently transformed lines of the potato cultivar Katahdin carrying the *RB* transgene were made resistant to *P. infestans* US1, US8, and US11 in greenhouse tests. Subsequently, Kuhl and associates (2007) demonstrated that the potato breeding line MSE149-5Y transformed with *RB* was rendered resistant to *P. infestans* US1, US8, US10, and US14 in field and detached-leaf tests. However, neither 'Katahdin' nor MSE149-5Y rank among commercially prominent cultivars, and transgenic lines developed in those studies are of limited production utility. In greenhouse tests, Halterman and associates (2008) reported that the *RB* transgene imparts foliar blight resistance against *P. infestans* US8 to transformed potato cultivars Dark Red Norland, Katahdin, Russet Burbank, and Superior. In that study, a single transgenic line was tested for each cultivar. Until now, tests of agriculturally significant cultivars transformed with *RB* under field production conditions have not been reported.

Previously, we developed polymerase chain reaction (PCR) and reverse transcription (RT)-PCR assays specific for the *RB* transgene (Millett and Bradeen 2007). Capitalizing upon single nucleotide polymorphisms, our assays are so specific that they can differentiate between the *RB* transgene and the native gene from which it originated.

In this study, we examine the effects of the *RB* transgene on foliar late blight caused by *P. infestans* US8 in a range of commercially prominent potato cultivars under field conditions favorable for disease development. Resistance phenotypes are interpreted relative to quantitative measurements of *RB* tran-

script levels in the foliage and estimates of *RB* copy numbers. Our research demonstrates that the *RB* transgene can be used effectively to reduce foliar late blight in cultivated potato. *RB* originates from a relative of potato and a deployment strategy in which the gene is inserted into commercially prominent potato cultivars without the use of a selectable marker, a cis-genic approach, should be possible. Our results demonstrate that manipulation of *RB*-mediated resistance levels is possible within the context of cisgenic deployment.

RESULTS

Phenotypic evaluations.

Field evaluations revealed that the *RB* transgene imparts to cultivated potato meaningful levels of resistance to foliar late blight in the absence of fungicides (Fig. 1; Table 1). Resistance data were analyzed as the disease rating score (1 to 9 scale) on the last day of assessment, a measure of resistance phenotype, and as relative area under the disease progress curve (RAUDPC), a measure of the rate of disease progression. These measurements are not independent. Individual genotypes were also assigned to arbitrarily defined phenotypic disease-resistance categories based on final disease rating score. Among 57 transgenic lines, 13 (23%) were ranked as resistant and 26 (46%) as moderately resistant (Table 1). Under standard growing conditions in the United States, these resistance levels should ensure that late blight disease will not have a major impact on yield. Independent of cultivar, the presence of the *RB* transgene has a highly significant ($P < 0.01$) effect on disease development (Table 2).

Field data from 2005 and 2006 were strongly and significantly correlated (R^2 for RAUDPC = 0.68; $P < 0.01$), indicating that, on average, the *RB* transgene performed consistently in both years. Figure 2 illustrates RAUDPC in 2005 and 2006 for all transgenic and nontransgenic lines averaged across genotypes and separately for transgenic and nontransgenic

Fig. 1. A late blight nursery in Minnesota in 2005 photo taken approximately four weeks after inoculation of the field with *Phytophthora infestans* US8. The transgene *RB* imparts to potato agriculturally meaningful levels of resistance to foliar late blight under field conditions in the absence of fungicides. Transgenic plants (living) remain healthy and relatively disease free compared with nontransgenic potato cultivar NorChip (rows of dead, infected plants).

Table 1. Transgenic and nontransgenic potato lines evaluated for late blight resistance, *RB* copy number, and *RB* transcript levels

Line	Genotype	Callus ^a	Average RAUDPC ^b	Average final resistance rating ^c	Resistance ranking ^d	Resistance category ^e	Transgene copy number ^f	Transgene transcript level
'Dark Red Norland'	'Dark Red Norland'	n.a.	0.85 ± 0.02	9.00 ± 0.00		S	0	0.00
SP2511	'Dark Red Norland'+ <i>RB</i>	1,775	0.27 ± 0.03	3.16 ± 1.16	16	MR	2	0.06
SP2514	'Dark Red Norland'+ <i>RB</i>	1,776	0.62 ± 0.22	7.16 ± 2.22	52	MS	...	...
SP2515	'Dark Red Norland'+ <i>RB</i>	1,776	0.56 ± 0.12	6.50 ± 1.64	48	MS	1	0.03
SP2520	'Dark Red Norland'+ <i>RB</i>	1,779	0.37 ± 0.12	4.16 ± 1.32	31	MR	2 to 3	0.17
SP2531	'Dark Red Norland'+ <i>RB</i>	1,789	0.61 ± 0.10	7.16 ± 1.60	52	MS	1 to 2	0.07
SP2534	'Dark Red Norland'+ <i>RB</i>	1,786	0.53 ± 0.12	6.66 ± 1.36	49	MS	1	0.03
SP2561	'Dark Red Norland'+ <i>RB</i>	1,797	0.51 ± 0.10	6.33 ± 1.50	46	MS	1 to 2	0.02
SP2564	'Dark Red Norland'+ <i>RB</i>	1,798	0.29 ± 0.08	3.50 ± 1.04	18	MR	...	0.06
SP2565	'Dark Red Norland'+ <i>RB</i>	1,799	0.38 ± 0.10	4.50 ± 1.51	34	MR	5	0.08
SP2572	'Dark Red Norland'+ <i>RB</i>	1,803	0.42 ± 0.12	5.33 ± 1.75	42	MS	...	0.07
SP2573	'Dark Red Norland'+ <i>RB</i>	1,803	0.50 ± 0.16	6.00 ± 1.41	45	MS	1 to 2	...
SP2574	'Dark Red Norland'+ <i>RB</i>	1,803	0.39 ± 0.08	4.83 ± 1.60	39	MR	3 to 4	0.01
SP2575	'Dark Red Norland'+ <i>RB</i>	1,804	0.31 ± 0.06	3.66 ± 1.36	21	MR	2 to 3	0.12
SP2576	'Dark Red Norland'+ <i>RB</i>	1,804	0.29 ± 0.07	3.50 ± 1.22	18	MR	2 to 3	0.11
SP2577	'Dark Red Norland'+ <i>RB</i>	1,804	0.32 ± 0.06	3.66 ± 1.50	21	MR	2 to 3	0.02
SP2582	'Dark Red Norland'+ <i>RB</i>	1,805	0.57 ± 0.12	7.00 ± 1.67	51	MS	1 to 2	...
SP2585	'Dark Red Norland'+ <i>RB</i>	1,807	0.48 ± 0.13	5.83 ± 1.32	44	MS	1	0.04
SP2586	'Dark Red Norland'+ <i>RB</i>	1,807	0.51 ± 0.08	6.33 ± 1.21	46	MS	1	...
SP2587	'Dark Red Norland'+ <i>RB</i>	1,807	0.29 ± 0.06	3.66 ± 1.36	21	MR	3	0.04
SP2588	'Dark Red Norland'+ <i>RB</i>	1,808	0.38 ± 0.09	5.00 ± 1.67	41	MS	2 to 3	0.09
SP2619	'Dark Red Norland'+ <i>RB</i>	1,779	0.19 ± 0.07	2.00 ± 0.00	4	R	3	...
SP2633	'Dark Red Norland'+ <i>RB</i>	1,788	0.37 ± 0.08	5.00 ± 1.41	41	MS	3	0.03
SP2654	'Dark Red Norland'+ <i>RB</i>	1,798	0.19 ± 0.03	2.33 ± 0.57	11	R	4-5	...
SP2658	'Dark Red Norland'+ <i>RB</i>	1,799	0.29 ± 0.02	3.5 ± 0.70	18	MR	...	...
SP2662	'Dark Red Norland'+ <i>RB</i>	1,803	0.30 ± 0.04	4.00 ± 0.89	27	MR	2 to 3	...
SP2663	'Dark Red Norland'+ <i>RB</i>	1,803	0.46 ± 0.16	5.5 ± 1.04	43	MS	1	0.05
SP2664	'Dark Red Norland'+ <i>RB</i>	1,803	0.55 ± 0.09	6.66 ± 1.36	49	MS	1 to 2	0.04
SP2665	'Dark Red Norland'+ <i>RB</i>	1,804	0.34 ± 0.09	4 ± 1.26	27	MR	2 to 3	...
SP2671	'Dark Red Norland'+ <i>RB</i>	1,807	0.28 ± 0.08	3.83 ± 1.16	25	MR	3 to 4	0.09
'Katahdin'	'Katahdin'	n.a.	0.76 ± 0.04	8.16 ± 0.40		S	0	0.00
SP905	'Katahdin'+ <i>RB</i>	582	0.32 ± 0.05	3.83 ± 0.75	14	MR	1	0.00
SP918	'Katahdin'+ <i>RB</i>	587	0.26 ± 0.01	2.66 ± 0.51	4	MR	1 to 2	0.10
SP922	'Katahdin'+ <i>RB</i>	587	0.31 ± 0.05	3.16 ± 0.98	1	MR	2 to 3	0.08
SP946	'Katahdin'+ <i>RB</i>	596	0.86 ± 0.10	9.00 ± 0.00	25	S	...	...
SP951	'Katahdin'+ <i>RB</i>	599	0.38 ± 0.08	4.4 ± 0.89	14	MR	1 to 2	0.04
SP966	'Katahdin'+ <i>RB</i>	604	0.41 ± 0.06	4.33 ± 1.03	16	MR	1	0.04
SP998	'Katahdin'+ <i>RB</i>	616	0.80 ± 0.06	8.66 ± 0.57	55	S	...	...
SP1453	'Katahdin'+ <i>RB</i>	1,244	0.23 ± 0.05	2.66 ± 0.81	33	MR	...	0.26
SP1464	'Katahdin'+ <i>RB</i>	1,249	0.20 ± 0.03	2.00 ± 1.00	32	R	3	0.28
SP1466	'Katahdin'+ <i>RB</i>	1,250	0.20 ± 0.07	1.50 ± 0.70	54	R	...	...
'Russet Burbank'	'Russet Burbank'	n.a.	0.97 ± 0.02	9.00 ± 0.00		S	0	0.00
SP2105	'Russet Burbank'+ <i>RB</i>	1,585	0.19 ± 0.03	2.16 ± 0.98	8	R	3	0.44
SP2113	'Russet Burbank'+ <i>RB</i>	1,593	0.19 ± 0.01	2.16 ± 0.40	8	R	15	0.14
SP2174	'Russet Burbank'+ <i>RB</i>	1,575	0.22 ± 0.04	3.66 ± 0.57	21	MR	2	0.17
SP2178	'Russet Burbank'+ <i>RB</i>	1,576	0.39 ± 0.07	4.50 ± 1.37	34	MR	1	0.02
SP2182	'Russet Burbank'+ <i>RB</i>	1,578	0.20 ± 0.03	2.00 ± 0.00	4	R	...	0.73
SP2184	'Russet Burbank'+ <i>RB</i>	1,578	0.23 ± 0.02	2.16 ± 0.40	8	R	2 to 3	0.02
SP2193	'Russet Burbank'+ <i>RB</i>	1,585	0.27 ± 0.04	2.33 ± 0.51	11	R	2	0.04
SP2210	'Russet Burbank'+ <i>RB</i>	1,593	0.19 ± 0.02	1.66 ± 0.51	2	R	...	0.06
SP2211	'Russet Burbank'+ <i>RB</i>	1,593	0.18 ± 0.02	1.83 ± 0.40	3	R	15	0.56
SP2212	'Russet Burbank'+ <i>RB</i>	1,594	0.34 ± 0.09	4.00 ± 1.78	27	MR	1	0.02
SP2213	'Russet Burbank'+ <i>RB</i>	1,594	0.21 ± 0.02	2.00 ± 0.00	4	R	...	...
SP2215	'Russet Burbank'+ <i>RB</i>	1,595	0.21 ± 0.02	2.33 ± 0.57	11	R	...	...
'Superior'	'Superior'	n.a.	0.91 ± 0.03	9.00 ± 0.00		S	0	0.00
SP2354	'Superior'+ <i>RB</i>	1,722	0.98 ± 0.02	9.00 ± 0.00	55	S	2	0.03
SP2361	'Superior'+ <i>RB</i>	1,724	0.93 ± 0.02	9.00 ± 0.00	55	S	...	...
SP2366	'Superior'+ <i>RB</i>	1,727	0.31 ± 0.04	4.00 ± 0.89	27	MR	4	0.03
SP2423	'Superior'+ <i>RB</i>	1,745	0.44 ± 0.09	4.60 ± 0.89	36	MR	2	...
SP2424	'Superior'+ <i>RB</i>	1,745	0.41 ± 0.11	4.66 ± 1.21	37	MR	2 to 3	...
SP2466	'Superior'+ <i>RB</i>	1,767	0.39 ± 0.07	4.66 ± 1.21	37	MR	2	0.01

^a n.a. = not applicable; genotype did not originate from callus.^b Relative area under the disease progress curve (RAUDPC) averaged across all replicates and both years ± standard deviation.^c Final resistance rating averaged across all replicates and both years ± standard deviation.^d Resistance ranking (from most resistant to least resistant) of transgenic lines based on average final resistance rating. Lines with identical average final resistance ratings are assigned to the same rank.^e Resistance categories: S = susceptible, MS = moderately susceptible, MR = moderately resistant, and R = resistant.^f Transgene copy numbers are estimated to the nearest whole number ±0.25. When a range is specified, quantitative polymerase chain reaction (PCR) values fell between the values specified (e.g., the range "1 to 2" indicates that the observed quantitative PCR value was more than 1.25 but less than 1.75). "..." indicates that no estimates were made.

lines of each genotype. Lines carrying the *RB* transgene displayed lower RAUDPC in all genotypes in each year. RAUDPC plots are consistent with reports that *RB* does not fully prevent disease but rather suppresses the rate of disease development (Song et al. 2003). Averaged across genotypes, RAUDPC values were virtually identical in 2005 and 2006. However, modest differences between RAUDPC values from 2005 and 2006 can be noted for specific genotypes (e.g., 'Katahdin'; Fig. 2). Consistent with this observation, year was a significant ($P < 0.01$) factor in analysis of variance (ANOVA) based on RAUDPC and final disease rating (Table 2). However, the interaction of the *RB* transgene with year did not contribute significantly ($P > 0.05$) to the final resistance rating but did contribute significantly ($P < 0.05$) to RAUDPC (Table 2). Taken together, our findings indicate that the *RB* transgene imparts a meaningful level of resistance that may be impacted to a modest degree by year-to-year variations. However, in both years, disease resistance levels of the transgenic lines far exceeded those of nontransgenic controls for all cultivars (Figs. 1 and 2).

ANOVA revealed that potato genotype has a significant ($P < 0.01$) effect on both RAUDPC and final resistance rating (Table 2). Thus, genetic background, independent of the *RB* transgene, impacts late blight resistance. Importantly, the interaction between the *RB* transgene and genotype was also significant ($P < 0.01$; Table 2). However, these results must be interpreted in light of our experimental approaches. For transgenic lines of the genotypes 'Russet Burbank' and 'Katahdin', greenhouse analyses of late blight resistance were conducted first and the results used to select predominantly resistant lines for field evaluation. For 'Superior' and 'Dark Red Norland', field evaluations of transgenic lines were conducted independently of greenhouse analyses. Field and greenhouse data were highly and significantly correlated ($R^2 = 0.67$, $P < 0.01$; Fig. 3). Our results suggest that foliar late blight resistance data from greenhouse tests of whole plants accurately predict resistance phenotypes in field plots. Utilizing greenhouse resistance data to select transgenic 'Russet Burbank' and 'Katahdin' for subsequent field tests skewed these lines toward higher resistance levels and, thus, impacted the validity of the genotype factor and its interaction with the presence of the *RB* transgene in our ANOVA results. Further analysis on the effects of genotype on late blight resistance in the presence of the *RB* transgene is warranted.

Transgenic line within the interaction between *RB* and genotype was a significant ($P < 0.01$) factor in ANOVA results (Table 2). Observations of line-to-line differences in late blight resistance suggest that characterization of a moderately large

number of transgenic lines to identify lines with the best combination of late blight resistance and agronomic performance is an effective first step toward *RB* deployment.

Molecular evaluations.

We next turned our attention to exploration of *RB* transgene copy number and transcript levels. The PCR and RT-PCR assays we utilized have a high degree of specificity for the *RB* transgene (Millett and Bradeen 2007). Accordingly, in no PCR or RT-PCR was template detected in nontransformed potato genotypes. Once adapted to real time quantification, our assays were applied to each of the transgenic lines. Our results indicate that *RB* copy number and transcript levels vary substantially across transgenic lines (Table 1).

Quantitative real time PCR has been advocated for accurate estimation of transgene copy numbers (Bubner and Baldwin 2004; Ingham 2005; Ingham et al. 2001; Lovatt 2002). Bubner and Baldwin (2004) and Yuan and associates (2007) reviewed approaches and statistical models for validating real time PCR estimated transgene copy numbers. In this study, *RB* copy numbers were estimated using two different standards for normalization, i.e., *Elongation Factor 1- α* (*EF1- α*) and *Urease*. *EF1- α* is an appropriate standard for RT-PCR characterization of potato-*P. infestans* interactions (Nicot et al. 2005). *Urease* is a single copy gene in potato (Witte et al. 2005). *RB* copy numbers normalized to these two standards were highly and significantly correlated ($R^2 = 0.71$, $P < 0.01$), strengthening our confidence in our quantitative PCR estimates. In subsequent analyses, we used *RB* copy number estimates normalized against *Urease*. Additionally, seven transgenic lines evaluated in this study were independently assayed for *RB* transgene copy number via Southern hybridization (Kramer et al. 2009). Results were concordant, indicating that these methods are equally reliable for estimating copy numbers. Nevertheless, regardless of the method employed, estimates of *RB* transgene copy number should only be interpreted as approximate (Bubner and Baldwin 2004).

Quantitative PCR was employed to estimate *RB* transgene copy numbers for 44 of the 57 transformed lines characterized for foliar late blight resistance. Across all cultivars, the average *RB* transgene copy number per line is 2.78, but averages varied between cultivars from 1.84 copies for 'Katahdin' to 5.25 copies for 'Russet Burbank'. While 91% (40/44) of the transformed lines have been estimated to have four or fewer transgene copies per genome, two outliers, SP2113 and SP2211, have an estimated 15.07 and 15.08 copies, respectively. Both of these lines are derived from 'Russet Burbank' and originate from a common callus (#1593; Table 1). Similar estimates of copy

Table 2. Analysis of variance of potato foliar late blight field resistance data

Source	Df ^b	RAUDPC ^a		Final resistance rating	
		Mean square	F value ^c	Mean square	F value
Genotype	3	0.736	194.16**	100.882	133.29**
<i>RB</i> (presence of transgene)	1	5.068	1,336.59**	435.908	575.93**
Year	1	0.681	179.58**	97.842	129.27**
<i>RB</i> × Genotype	3	0.155	40.77**	10.193	13.47**
Line within (<i>RB</i> × Genotype)	53	0.113	29.80**	12.328	16.29**
Year × Genotype	3	0.103	27.30**	19.440	25.68**
<i>RB</i> × Year	1	0.016	4.33*	...	...
Year × Line within (<i>RB</i> × Genotype)	42	0.010	2.70**	1.475	1.95**
<i>RB</i> × Year × Genotype	3	0.011	2.81*	...	...
Error	217	0.004		0.084	

^a RAUDPC = relative area under the disease progress curve.

^b DF = degrees of freedom.

^c * = significant at $0.05 < P < 0.01$; ** = significant at $P < 0.01$.

^d Nonsignificant ($P > 0.05$) two- and three-way interactions were omitted.

number, similar phenotypic performance, and their origin from a common callus suggest that lines SP2113 and SP2211 may derive from a single transformation event. We plotted average final late blight resistance ratings against the corresponding

average estimated transgene copy numbers for the four disease resistance categories (resistant, moderately resistant, moderately susceptible, and susceptible). A clear trend of enhanced disease resistance with increasing transgene copy numbers

Fig. 2. *RB* suppresses but does not eliminate foliar late blight development in a range of commercially viable potato genotypes under field conditions in the absence of fungicides. Plots of the relative area under the disease progress curve (RAUDPC) for transgenic (+*RB*) and nontransgenic (–*RB*) potato vs. number of days postinoculation with *Phytophthora infestans* US8 in 2005 and 2006. **A**, RAUDPC averaged across genotypes and lines; **B**, RAUDPC averaged across lines for the genotype ‘Dark Red Norland’; **C**, RAUDPC averaged across lines for the genotype ‘Katahdin’; **D**, RAUDPC averaged across lines for the genotype ‘Russet Burbank’; **E**, RAUDPC averaged across lines for the genotype ‘Superior’. Dotted lines indicate data for nontransgenic (–*RB*) lines from 2005; dashed lines indicate data for nontransgenic (–*RB*) lines from 2006; solid lines indicate data from transgenic (+*RB*) lines in 2005; dash-dotted lines indicate data from transgenic (+*RB*) lines in 2006. Note that while disease develops in both transgenic potato and untransformed controls, the rate of disease development is substantially faster in nontransgenic potato. This is true for each genotype examined. Also note correspondence between data from 2005 and 2006, especially in panel A. Data are strongly correlated ($R^2 = 0.678$).

emerged (Fig. 4). Average copy numbers per resistance category ranged from 1.17 copies for the susceptible class (based on a single observation) to 3.00 ± 1.61 for the resistant class (outliers SP2113 and SP2211 were removed from this calculation). Nevertheless, highly resistant lines carrying few transgene copies (e.g., SP2193, a resistant ‘Russet Burbank’ line with approximately two *RB* transgene copies; Table 1) can be identified despite the observed trend of increasing resistance with increasing transgene copy number. Consistently, correlation between final foliar blight resistance rating and estimated transgene copy number was weak but significant ($R^2 = 0.191$, $P < 0.01$).

Finally, we explored transcript levels of the *RB* transgene. *RB* transcript levels were normalized to *EFL-α*, an appropriate standard for potato–*P. infestans* interactions (Nicot et al. 2005). *RB* transcript levels were determined for 40 transgenic lines. Similar to what was observed for estimated transgene copy numbers, a trend of enhanced foliar blight resistance with increasing transgene transcript levels was evident (Fig. 4). However, average transcript levels for each resistance class varied considerably, as evident by large standard deviations (Fig. 4). Transcript levels were weakly but significantly correlated with final resistance rating ($R^2 = 0.193$, $P < 0.01$). Importantly, estimated transgene copy numbers were also significantly correlated with transcript levels ($R^2 = 0.349$, $P < 0.01$).

‘Russet Burbank’ lines SP2113 and SP2211, each of which contain an estimated 15 *RB* transgene copy numbers, deserve special mention. These ultra-high copy number lines have the highest *RB* copy number of 44 lines surveyed (Table 1). These lines are also among the most late blight resistant, ranking as the third (SP2211) and eighth (SP2113) most late blight resistant lines of 57 transgenic lines surveyed in our field study. Similarly, transcript levels in lines SP2113 and SP2211 are high. Of 40 lines surveyed, SP2113 and SP2211 rank as the lines with the eighth and second highest transcript levels, respectively. Importantly, since both transcript levels and phenotypic resistance remain high in SP2113 and SP2211, our results suggest no evidence that the ultra-high *RB* transgene copy numbers associated with these lines have triggered innate RNA silencing mechanisms that could effectively silence the transgenes.

DISCUSSION

More than 150 years after the Irish potato famine, late blight of potato still ranks among the world’s most economically damaging plant diseases, necessitating frequent preventative fungicide applications in regions of the world prone to the disease. Genetic resistance to late blight is an economically and environmentally friendly alternative to chemicals. In this study,

Fig. 3. Greenhouse tests of foliar late blight resistance predict field performance of potato genotypes transformed with *RB*. Plot of final foliar late blight resistance ratings from greenhouse and field assays. Individual points represent average data for each of 46 potato lines carrying the *RB* transgene; data were averaged across years and replicates. Note that visual rating scales used in field and greenhouse trials are different. For reference, phenotypic resistance categories (R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible) based on average percent foliar infection are shown. Lines delineate resistance categories. Shaded regions of the graph indicate concordance in resistance categories between greenhouse and field assays. Of 46 transgenic lines, 24 were assigned to the same resistance category by both assays, and results of the two assays are highly correlated ($R^2 = 0.67$).

we have demonstrated that the transgene *RB*, derived from a wild relative of cultivated potato, functions in a range of commercially prominent potato cultivars to impart agriculturally meaningful levels of resistance under field conditions, even in the absence of fungicides. In total, 39 of 57 lines tested were rated as resistant or moderately resistant, suggesting that a transgenic approach to incorporating *RB* into commercially viable potato cultivars has promise for direct production application.

The near future of transgenic potato in the United States is uncertain, since processor concerns have limited the marketability of transgenic potatoes (Bradeen et al. 2008). But as studies demonstrate that transgenic potatoes do not differ significantly from untransformed potatoes at the proteome (Lehesranta et al. 2005) and metabolome (Catchpole et al. 2005) levels and as grower and environmental costs associated with frequent fungicide applications are more carefully scrutinized, transgenic approaches may gain wider acceptance. Towards this goal, the cisgenic movement advocates for distinction between transgenes, moved between distantly related species, and cisgenes, incorporated via transformation from a closely related species. *RB* originates from a wild relative of potato and, within the context of deployment in cultivated potato, may therefore be considered a cisgene. Recent research suggests the distinction between transgene and cisgene may influence consumer opinion (Lusk and Sullivan 2002). Given the worldwide significance of potato as a food crop and potato late blight as a limiting production factor, the cisgenic deployment of *RB* may be a feasible approach for utilization. Conversely, *RB* may be an ideal test case for the use of cisgenics in potato improvement.

In this study, comparison of transgene copy numbers, transgene transcript levels, and phenotypic resistance revealed a generalized trend of enhanced resistance with increasing transgene copy numbers and higher transcript levels (Fig. 4). Our results suggest that researchers may achieve transgenic lines with superior resistance by increasing *RB* transcription. As reflected in Figure 4, one approach to achieve this may be to insert additional copies of the *RB* transgene. Consistent with this conclusion, scientists working in a range of plant species have previously noted direct correlations between transgene copy numbers and transcript accumulation in some instances

(Hobbs et al. 1993; Ku et al. 1999; Schubert et al. 2004; Stockhaus et al. 1987). However, both *RB* transcript levels and copy numbers varied substantially among individual lines within a resistance category (Fig. 4), suggesting that it is possible to identify highly resistant lines with relatively few transgene copies.

Our observations of a positive correlation between transgene transcript levels and phenotypic resistance are not without precedence. Cao and associates (2007) demonstrated that the rice *Xa3* resistance gene imparts resistance against *Xanthomonas oryzae* pv. *oryzae* in a genetic background-dependent fashion, with higher disease resistance levels observed in a *japonica* background than in an *indica* background. Importantly, *Xa3* transgene transcript levels were directly correlated with observed resistance. Up- or downregulation of the *Xa3* transgene, achieved in that study by switching promoters, led to enhanced or reduced disease resistance, respectively (Cao et al. 2007). Thus, for both *RB* and *Xa3*, enhancing transgene transcription might be an effective means of increasing disease resistance. However, Cao and associates (2007) also demonstrated that *Xa3* imparts resistance in a developmental stage-dependent fashion that is also correlated with transcript levels. Specifically, young rice plants showed both reduced *Xa3* transcript levels and reduced resistance relative to adult plants. In potato, we recently demonstrated that *RB*-mediated late blight resistance varies throughout plant development, with young, pre-flowering plants being more disease resistant than post-flowering or near senescing plants (Millett et al. 2009). Contrary to what was observed for *Xa3*, however, we demonstrated no significant difference in *RB* transgene transcript levels throughout plant development. Comparison of the transcriptional behavior of *RB* and *Xa3* suggests that both commonalities and differences exist between *R* genes in modes of functional regulation.

We measured transcript levels of the *RB* transgene in uninfected tissue prior to inoculation with *P. infestans*. The detection of the *RB* transcript in these materials confirms that, like the lettuce *R* gene *Dm3* (Shen et al. 2002), *RB* is transcribed constitutively. Kramer and associates (2009) examined *RB* transcript levels in transgenic 'Katahdin' plants prior to and for several days after infection with *P. infestans*. They noted a

Fig. 4. Increasing *RB* transgene copy numbers and transcript levels correspond with a trend towards enhanced foliar late blight resistance. **A**, Estimated transgene copy number averaged across all genotypes vs. disease resistance categories (R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible) based on final disease resistance rating. Error bars are standard deviations about means. A single observation was available for the S category. As average transgene copy number increases, a corresponding increase in average disease resistance is evident. **B**, Normalized transgene transcript levels averaged across all genotypes vs. disease resistance categories based on final disease resistance rating. Error bars are standard deviations about means. A single observation was available for the S category. As average transcript levels increase, a corresponding increase in average disease resistance is evident. Note that transcript levels are more variable than average transgene copy number, especially for the R category, as evident by comparatively larger standard deviations. Lines SP2113 and SP2211, each with an estimated 15 *RB* copies, were excluded from this analysis. Exclusion of these materials did not alter data trends.

clear increase in *RB* transcription in response to pathogen attack. Pathogen-induced upregulation of *R* genes has been noted in other pathosystems in some instances (Gu et al. 2005; Halterman et al. 2003; Levy et al. 2004; Thureau et al. 2003).

Interestingly, high copy numbers of the *RB* transgene appear not to trigger innate RNA silencing mechanisms. Two transgenic lines, SP2113 and SP2211, each have an estimated 15 copies of the *RB* transgene. Despite these ultra-high copy numbers, both transcript levels and disease resistance remain high in these lines (Table 1). (The possibility that lines SP2113 and SP2211 arose from a common transformation event warrants further study.) Similar to what we observed in this study, after comparing transcript levels in 132 independent transformed lines of *Arabidopsis*, Schubert and associates (2004) concluded that transgene transcript levels and copy numbers are positively correlated, up to a certain level. However, beyond a certain number of transgene copies, RNA silencing was evident. Most importantly, Schubert and associates (2004) concluded that RNA silencing of transgenes occurs only when transcript levels pass a gene-specific threshold, achieved in that study by the introduction of more transgene copies, each under the control of the strong *Cauliflower mosaic virus* 35S promoter. In this study, the *RB* transgene is under the control of its native, endogenous promoter. Relative to our RT-PCR standard *EF1- α* , the transgenic lines accumulate only low to modest levels of the *RB* transcript (Table 1; Fig. 4). Consistent with this observation, based on band intensities revealed via Northern hybridizations, Parker and associates (1997) concluded that the *Arabidopsis R* gene *RPP5* is transcribed at very low levels. Similarly, Shen and associates (2002) were unable to detect transcript of the lettuce *R* gene *Dm3* in Northern hybridizations of total RNA and concluded that this gene, too, is transcribed at low levels. Thus, transcripts of *R* genes in general may accumulate in the plant cell at relatively low levels. Our results with the *RB* transgene suggest that the low levels of *R*-gene transcript accumulation mediated by endogenous promoters do not fully engage RNA silencing mechanisms, even in plants containing ultra-high transgene copy numbers.

Late blight continues to be a devastating disease for potato producers. The cloning of broad-spectrum genes conferring a pathogen rate-reducing phenotype, like *RB*, now offers the possibility of using transgenic technologies for potato improvement. Phenotypic and molecular results presented in this study demonstrate that transformation of commercially prominent potato cultivars with the *RB* transgene is an effective means of imparting genetic disease resistance. Additional research aimed at understanding the biological and molecular function of *RB* and the impact of plant genotype, physiological age, and organ on *RB* function will prove essential to integrating *RB* into a production system that favors the long-term effectiveness of the gene.

MATERIALS AND METHODS

Plant materials.

Potato cultivars Dark Red Norland, Katahdin, Russet Burbank, and Superior were transformed with the *RB* construct using *Agrobacterium*-mediated transformation, as previously described (Song et al. 2003). The *RB* transgene used in these experiments consists of an 8.5-kb long-range PCR fragment isolated from *S. bulbocastanum* genotype PT29 genomic DNA. This fragment incorporates the entire coding region of the gene, a single intron, and the native promoter. For each potato cultivar, multiple transgenic lines were generated (Table 1). Transgenic lines originating from a common callus may or may not represent independent transformation events. Only lines testing positive for the transgene (verified by PCR as pre-

viously described [Song et al. 2003]) were advanced to phenotypic evaluations. Minitubers were produced for each transgenic line by planting in vitro plantlets in standard potting medium in 4-in. pots. Plantlets were maintained in a mist house with frequent misting to aid in root development for approximately 2 weeks prior to growth to maturity under standard greenhouse conditions in St. Paul, MN. Minitubers were harvested and stored for a minimum of 2 months at 4°C to break physiological dormancy. All transgenic lines tested in this study are maintained in vitro or as greenhouse-grown minitubers at the University of Minnesota or University of Wisconsin; germplasm requests should be directed to J. Jiang.

Field phenotypic evaluations.

Minitubers of transgenic lines and untransformed controls were planted in a late blight nursery at the University of Minnesota UMore Park, Rosemount, MN, U.S.A. on June 15, 2005 and June 7, 2006. Each experimental unit consisted of a four-hill plot, with tubers planted on 0.3-m centers. Each transgenic line was replicated three times (three independent experimental units) in each year. Lines were randomized within replicates. All experimental lines were planted between rows of cultivated potato 'Norchip', a variety known to be foliar late blight susceptible. No fungicide was applied to the nursery in either test year.

Inoculum of *P. infestans* US8 (isolate 940480 [A2]) was prepared by culture on rye A agar (Caten and Jinks 1968) at 16°C for 1 month. Sporangia and mycelia were washed from the plates and were inoculated onto cuttings of late blight-susceptible potato maintained under cool, humid conditions as described by Millett and associates (2009). Inoculum was thus cycled through two rounds of increase on plant material prior to field inoculation. Prepared inoculum was incubated for 60 min at 4°C, followed by 30 min at room temperature, before being transported to the research field plot on ice. On the evenings of August 15, 2005 and August 8, 2006, the *P. infestans* inoculum was applied by hand with the assistance of a CO₂ sprayer to all 'Norchip' plants in the research field at a concentration of approximately 7,000 sporangia per linear meter of row. Subsequent frequent (approximately 2 h every other day) overhead irrigation created high humidity conditions favorable for the development and spread of the pathogen within the research field. Visual assessments of disease development were made in all experimental plots twice per week for 1 month following inoculation. Visual assessments utilized the 1 to 9 scale of Henfling (1987), in which 1 = no disease, 2 = less than 5% of the foliage is diseased, 3 = 5% to less than 15% of the foliage is diseased, 4 = 15% to less than 35% of the foliage is diseased, 5 = 35% to less than 65% of the foliage is diseased, 6 = 65% to less than 85% of the foliage is diseased, 7 = 85% to less than 95% of the foliage is diseased, 8 = 95% to less than 100% of the foliage is diseased, and 9 = 100% of the foliage is diseased. For purposes of comparison, average final disease resistance ratings were used to assign potato lines to resistance phenotype classes. Thus, genotypes scoring less than 2.5 were considered resistant, genotypes scoring 2.5 to less than 5.0 were considered moderately resistant, genotypes scoring 5.0 to less than 7.5 were considered moderately susceptible, and genotypes scoring more than 7.5 were considered susceptible.

Greenhouse phenotypic evaluations.

For 46 transgenic lines evaluated in field plots at the University of Minnesota, foliar late blight resistance was also evaluated in greenhouses at the University of Wisconsin, using previously reported procedures (Halterman et al. 2008; Song et al. 2003). Notable deviations from field-based methods include the use of an independent isolate of *P. infestans* US8 and use

of an inverted scale for visual ratings. Greenhouse evaluations were independent of field evaluations for transgenic lines originating from 'Dark Red Norland' and 'Superior'. For 'Katahdin' and 'Russet Burbank', greenhouse evaluations were conducted first, and only those transgenic lines displaying some level of resistance were advanced to field trials. For purposes of comparison, average final disease resistance ratings from greenhouse tests were used to assign potato lines to resistance phenotype classes. Thus, genotypes scoring 8.0 or above in greenhouse tests were considered resistant, genotypes scoring 5.0 to less than 8.0 were considered moderately resistant, genotypes scoring 1.5 to less than 5.0 were considered moderately susceptible, and genotypes scoring less than 1.5 were considered susceptible. These designations are based on approximate percent foliage infected and greenhouse resistance categories are directly comparable with field resistance categories.

Estimation of *RB* transgene copy numbers.

To quantify transgene copy numbers, DNA was isolated from leaves harvested from greenhouse-grown plants of each transgenic line using the DNeasy plant mini kit (Qiagen Inc., Valencia, CA, U.S.A.) and manufacturer's protocol. DNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, LLC, Wilmington, DE, U.S.A.) and was stored at -20°C . *RB* transgene-specific primers 2MAMA5'3 (forward, TTGCAACCATATGAACTGTCAACC) and 2MAMA3'1 (reverse, CCAGAGAGAGATTAGCTTTTCTTTTCT) were reported previously (Millett and Bradeen 2007) and were used to develop a real time PCR assay to estimate *RB* transgene copy numbers. Our optimized assay utilized the iTaq SYBR Green supermix with ROX (Bio-Rad, Hercules, CA, U.S.A.) in a 25- μl total volume, following manufacturer's recommendations, and 5 μl (10 to 50 ng) of template. Reactions were assayed in an Applied Biosystems 7500 Real-Time PCR system (Foster City, CA, U.S.A.). *RB* copy numbers were normalized relative to *EF1- α* and *Urease*. *EF1- α* primers *EF1-f* (forward, ATTGGAACGGATATGCTCCA) and *EF1-r* (reverse, TCCTTACCTGAACGCCTGTCA) were designed by Nicot and associates (2005). For *Urease*, we employed primers *Ure-f1* (Fw GACCTGTTTGCTGAAATTGAGA) and *Ure-r1* (Rv GAACTTTTCCACCCCAAAC). All primers were obtained from Integrated DNA Technologies (Coralville, IA, U.S.A.). *RB* primers were used at concentrations of 200 (forward) and 900 nM (reverse), *EF1- α* primers were used at a concentration of 400 nM, and *Urease* primers were used at a concentration of 300 nM. *RB*, *EF1- α* , and *Urease* reactions were completed in separate tubes. All quantitative PCR assays underwent an initial denaturing step (2 min at 94°C). Reactions evaluating *RB* continued with 40 cycles of 94°C for 15 s, 57°C for 28 s, and 72°C for 32 s. Internal control *EF1- α* and *Urease* reactions used 40 cycles of 94°C for 15 s and 60°C for 60 s. All reactions were completed in triplicate and concluded with a melting curve evaluation. Results were evaluated using Sequence Detection Software v. 1.4.0.25 (Applied Biosystems). The threshold value was adjusted against control reactions for consistent cycle threshold (C_t) values across multiple experimental plates. C_t values for the *RB* transgene, *EF1- α* , and *Urease* were then processed with the Visual Basic software Q-Gen (Muller et al. 2002) in Microsoft Excel for Mac 2004 (v11.3.7, Microsoft Corp., Redmond, WA, U.S.A.).

Quantification of *RB* transcript.

For RNA analyses, the top-most, fully expanded leaf from one plant per transgenic line was harvested from field-grown plants immediately prior to inoculation on August 8, 2006. This leaf was immediately frozen in liquid nitrogen and was

stored at -80°C . RNA was extracted from a portion of this leaf using the SV total RNA isolation kit (Promega, Madison, WI, U.S.A.) according to manufacturer's recommendations and was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, LLC). Real time RT-PCR assays utilized the *RB* transgene-specific primers 2MAMA5'3 and 2MAMA3'1 (Millett and Bradeen 2007) and were performed as described previously (Millett et al. 2009). *RB* transgene transcript levels were normalized against *EF1- α* .

Data analyses.

All data, including visual ratings of disease resistance and real time PCR and RT-PCR data were compiled into an Excel spreadsheet. Excel was used to calculate RAUDPC and descriptive statistics including means, standard deviations, and correlations. Student *t*-test was employed to test the significance of correlations. ANOVA was performed using SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, U.S.A.). To explore and document the relationship between resistance data collected in the field and greenhouse and between resistance data and transgene copy number and transcript levels, scatter and bar plots were generated using Excel.

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