Performance of Transgenic Potato Containing the Late Blight Resistance Gene *RB*

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ABSTRACT

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Late blight of potato, caused by *Phytophthora infestans*, is one of the most devastating diseases of potato. A major late blight resistance gene, called *RB*, previously was identified in the wild potato species *Solanum bulbocastanum* through map-based cloning. The full-length gene coding sequence, including the open reading frame and promoter, has been integrated into cultivated potato (*S. tuberosum*) using *Agrobacterium*-mediated transformation. *RB*-containing transgenic plants were challenged with *P. infestans* under optimal late blight conditions in greenhouse experiments. All transgenic lines containing *RB* exhibited strong foliar resistance. Field-grown transgenic tubers also were tested for resistance to *P. infestans*. In contrast to the foliar resistance phenotype, *RB*-containing tubers did not exhibit increased resistance. Two years of field trials were used to ascertain whether the presence of *RB* had any effect on tuber yield. We were unable to detect any significant effect on tuber size or yield after addition of the resistance gene to several *S. tuberosum* cultivars.

Additional keywords: disease resistance

Phytophthora infestans, causal agent of potato and tomato late blight, remains one of the most destructive and costly of all plant pathogens worldwide (5,17,18). In the United States and other developed countries, management of late blight relies mostly on frequent application of fungicides because most cultivars of potato are highly susceptible. Developing potato cultivars with high levels of broad-spectrum resistance is a key long-term management strategy for combating this disease. Somatic fusions between *Solanum* spp. offer the opportunity to integrate resistance into cultivated potato (7). However, the process is laborious and the large number of undesirable traits from the wild species must be removed through several generations of backcrosses to *Solanum tuberosum* cultivars. An alternative strategy is the introduction of a single gene through stable transformation. The advantage of this procedure is that a highly desirable trait, conferred by one or a few genes, can be added with little chance of drastically altering the recipient plant's genetic struc-

ture or physiology under normal growth conditions. With the addition of a single resistance (*R*) gene, the resultant plant gains the ability to defend itself against a normally virulent pathogen, reducing the need for application of pesticides (4).

A number of wild, tuber-bearing Mexican potato species have been reported to carry valuable resistance to late blight, thus providing a potential source of resistance for breeding programs (1,6). Eleven race-specific *R* genes have been identified in the wild potato species *S. demissum* and introgressed into cultivated potato using classical breeding methods (12,22,25). Unfortunately, virulent races of *P. infestans* have rapidly overcome the majority of these 11 *R* genes in most potato-growing regions (5,25). A major late-blight-resistance gene previously was identified in the wild potato species *S. bulbocastanum* and integrated into cultivated potato, *S. tuberosum*, through somatic fusion (8). The resistance gene, called *RB*, subsequently was cloned and found to belong to the largest class of *R* genes, those that encode proteins with a nucleotide binding site and leucine rich repeats (NB-LRR; 19,23). A long-range polymerase chain reaction (PCR) product including the gene coding region of *RB*, as well as approximately 5 kb of upstream regulatory sequences, was stably integrated into *S. tuberosum* using *Agrobacterium*-mediated transformation (19). Somatic fusions and transgenic plants containing

this gene are highly resistant to late blight, even when tested under optimal late blight conditions using multiple pathogen isolates (3,8,19). One of the most interesting aspects of *RB* is that it confers resistance instead of immunity (19). This phenotype differs somewhat from potato cultivars containing *R* genes from *S. demissum*, which rapidly produce macroscopic necrotic lesions indicative of a classical hypersensitive response (24).

Recently, the *RB* gene was integrated into four popular U.S. potato cultivars: Katahdin, Superior, Dark Red Norland, and Russet Burbank (J. Bradeen and S. Austin-Phillips, *unpublished data*). In preliminary experiments, these transgenic lines consistently exhibited high levels of resistance to *P. infestans* under greenhouse inoculation conditions (J. Jiang and D. A. Halterman, *unpublished data*). Here, we report the partial resistance phenotype and field performance of *RB*-containing plants. Transgenic lines and nontransgenic controls were grown in unsprayed field trials in the Central Sands region of Wisconsin in order to analyze tuber size and yield. Transgenic and nontransgenic plants also were evaluated for foliar (greenhouse) and tuber resistance to *P. infestans*.

MATERIALS AND METHODS

Plant materials. Transformation of the Katahdin, Superior, Russet Burbank, and Dark Red Norland lines used in these experiments was done as described previously (19). A long-range PCR product containing the *RB* gene from *S. bulbocastanum*, under control of its native promoter and terminator, was used for *Agrobacterium*-mediated transformation of the potato cvs. Katahdin, Superior, Russet Burbank, and Dark Red Norland as in Song et al. (19). The transgenic lines used in these experiments were chosen because preliminary investigations showed that they consistently exhibited a high level of resistance in greenhouse assays (J. Jiang and D. A. Halterman, *unpublished data*). The following *RB* transgenic lines were used for the experiments described in this paper: Katahdin, SP951; Superior, SP2423; Russet Burbank, SP2105; and Dark Red Norland, SP2564. The presence of *RB* within transgenic plants was verified using PCR primer pairs within the 5' and 3' ends of

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the gene. Primers 1-5 (5'-CTC ATT TTA CCC CTA CAA-3') and 3-5 (5'-GCG TTT TGG ACC CTT TTA-3') were used to amplify the 5' end of the gene and primers cf1 (5'-TAA GCA TGA GTT GGA ATA ACT-3') and cr1 (5'-GCC AGT CTT CTC CTA TTC CCT-3') were used to amplify the 3' end of the gene. PCR was performed using Takara ExTaq DNA polymerase (TaKaRa Bio Inc., Japan) and 1 µl of genomic DNA at 0.1 µg/µl under the following conditions: 2 min at 96°C; followed by 30 cycles of 1 min at 96°C, 30 s at 53°C, and 1 min at 72°C; and then incubation for 5 min at 72°C. The presence of the 5' and 3' ends was verified by observing 619- or 840-bp products, respectively, after separation on a 1% agarose gel.

Foliar resistance evaluations. A greenhouse resistance assay was used to determine whether the transgenic plants were resistant to *P. infestans* according to Naess et al. (13). Tissue culture plantlets were transplanted to the greenhouse 3 to 4 weeks after subculture (day and night temperatures of 23 and 15°C, respectively, with 14 h of light). Plants at the six- to eight-leaf stage were used for inoculation. Different transgenic lines were inoculated on different dates. However, nontransformed parental checks as well as susceptible cv. Katahdin plants were inoculated alongside test plants to verify the ability of the sporangia and germinated zoospores to cause disease on each inoculation date. In each experiment, three replicate plants from each cultivar were arranged in a completely randomized design within the inoculation chamber. Inoculation experiments were replicated three times on different dates.

P. infestans isolate US-8 940480 (A2) was obtained from Barbara Baker at The University of California, Berkeley and contains avirulence genes 8 and 9. *P. infestans* was grown on Rye A medium in the dark at 15°C (2). Cultures were flooded

with sterile, distilled water and washes were combined to obtain a suspension of approximately 75,000 sporangia/ml. Sporangial suspensions were placed at 12°C for 1.5 to 3 h to induce zoospore release. At 1 to 3 h before inoculation, plants were placed in a closed greenhouse with a misting system that maintained greater than 90% relative humidity. Greenhouse temperatures were set at 23°C during the day and 15°C at night with 14 h of light. Plants were inoculated in the early evening near the time the lights shut off and the temperature began to drop to nighttime levels. Each plant was sprayed with approximately 3 ml of the sporangial suspension on both the underside and topside of the leaves. The greenhouse mist system maintained high relative humidity throughout the experiment. Disease readings were taken 4, 7, and 10 days after inoculation.

The late blight resistance score was determined by visual inspection of the plants. The score was determined based on the percentage of infected leaf tissue as follows: 0 = 100, 1 = >90, 2 = 81 to 90, 3 = 71 to 80, 4 = 61 to 70, 5 = 41 to 60, 6 = 26 to 40, 7 = 11 to 25, and 8 = <10% infected tissue (3).

Tuber resistance assays. Inoculum of *P. infestans* isolate US-8 940480 was prepared as described for the foliar assays and adjusted to a concentration of 30,000 sporangia/ml. Fresh, field-harvested tubers from 2005 and 2006 yield tests (see below) were washed and allowed to dry overnight. At least three tubers per clone from both 2005 and 2006 were punctured with a pipette tip three to six times (at least 2 cm apart), depending on the size of the tuber. The punctures were 2 mm wide by 6 mm deep and arrayed on one-half of the tuber. Tubers were inoculated at each of the puncture sites with 20 µl of the 30,000-sporangia/ml suspension. The number of inoculation sites was recorded. Incubation

chambers containing all replicates of each transgenic or nontransgenic cultivar were lined with wet paper towels and placed inside heavy-duty plastic bags to maintain high humidity. These chambers were placed in a dark 15°C incubator for 10 days.

The tuber periderm was removed carefully with a knife and the diameter of the brownish area surrounding each puncture was measured. The tuber then was cut at each inoculation site and the depth of the lesion was measured. The diameter (d) and depth (h) measurements were used to calculate the approximate volume of diseased tissue (half of an ellipsoid) using the formula $(4/3 \times \pi \times [(d/2)^2] \times [h/2])/2$, assuming the spread of the pathogen was equal in all directions on the surface of the tuber (width and length), but not into the tuber (depth). Individual lesion measurements on each tuber were analyzed individually. Means were separated using a least significant difference test at $P = 0.05$ using SAS version 9.1.3 (11).

Yield tests. Greenhouse transplants (16 June 2005) or small whole tubers (25 April 2006) were planted in Plainfield sandy loam soil (mixed, mesic, Typic Udipsamment) at the Hancock Agricultural Research Station in central Wisconsin in a randomized complete block design with three plot replications. There were five plants per replicated plot of each test line and controls. Plots were not sprayed with fungicide throughout the growing season; however, no symptoms of a naturally occurring late-blight infection were observed on any plants in the test fields during these experiments. Field trials were limited because adherence to Animal and Plant Health Inspection Service regulations required that the transgenic plants be sexually separated from other potato plants. Therefore, both transgenic and nontransgenic plants were deflowered throughout the growing season to prevent transgenic pollen from drifting to sexually receptive plants. No suitable large-scale potato-production facilities were available that were at least 400 m from sexually receptive potato plants, and deflowering on a large scale would have been extremely difficult. Although flower removal can reduce tuber yield on a species-specific level (9), both transgenic and nontransgenic plants were treated similarly, eliminating the possibility of a competitive effect on total yield. Tubers were hand harvested and manually separated into undersize and US#1 size (>4.8 cm in diameter) categories. Data were analyzed using a nonparametric analysis of variance (ANOVA) based on a rank transformation using the GLM procedure of SAS (11).

Table 1. Presence or absence of *RB* in transgenic lines of potato and greenhouse resistance scores of *Phytophthora infestans*-inoculated lines^a

Genotype	5' end present	3' end present	10-day late-blight score ^b
Katahdin without <i>RB</i>	No	No	1.2 ± 0.2
SP951 (Katahdin + <i>RB</i>)	Yes	Yes	7.0 ± 0.2
Superior without <i>RB</i>	No	No	1.2 ± 0.3
SP2423 (Superior + <i>RB</i>)	Yes	Yes	6.6 ± 0.3
Russet Burbank without <i>RB</i>	No	No	1.0 ± 0.3
SP2105 (Russet Burbank + <i>RB</i>)	Yes	Yes	7.2 ± 0.2
Dark Red Norland without <i>RB</i>	No	No	0.9 ± 0.3
SP2564 (Dark Red Norland + <i>RB</i>)	Yes	Yes	7.6 ± 0.2
<i>S. bulbocastanum</i> PT29	Yes	Yes	8.0 ± 0.0
Cultivar + <i>RB</i> ^c	...	...	7.1 ± 0.1
Cultivar without <i>RB</i>	...	...	1.1 ± 0.1

^a Verification of the presence of *RB* was performed using polymerase chain reaction with primers specific for the 5' and 3' ends of the transgene.

^b Average score among three replicates using the scale described in the methods section (0 = susceptible, 8 = resistant). Data shown is the mean late blight score ± the standard error.

^c When the average scores of all cultivars (except PT29) were combined, there were no significant date, cultivar, or cultivar-date interaction effects based on analysis of variance; the presence of *RB* was significant ($P < 0.0001$).

RESULTS

Assay of foliar resistance to *P. infestans* in four *RB*-containing *S. tuberosum* cultivars. Representatives of each

line were spray inoculated with *P. infestans* under humid greenhouse conditions as described in the Material and Methods section. All transgenic lines showed a high level of resistance to *P. infestans* (Table 1). Disease resistance ratings of SP951, SP2423, SP2105, and SP2564 averaged 7.0 (± 0.7), 6.6 (± 0.6), 7.2 (± 0.7), and 7.6 (± 0.5), respectively, 10 days after inoculation. *S. bulbocastanum* accession PT29 exhibited only some chlorosis on lower leaves and maintained an 8.0 (± 0.0) score throughout the experiment. In contrast to the *RB*-transformed lines, inoculation of nontransformed Katahdin, Superior, Russet Burbank, and Dark Red Norland plants resulted in late-blight-resistance scores of 1.3 (± 0.6), 1.2 (± 0.8), 1.0 (± 0.9), and 0.9 (± 0.8), respectively, 10 days after inoculation. When the average scores of all cultivars (except PT29) were combined, there were no significant date, cultivar, or cultivar-date interaction effects based on ANOVA. The presence of *RB* was significant ($P < 0.0001$).

The intermediate resistance phenotype of *RB*-containing plants was more pronounced in *RB*-transgenic *S. tuberosum* plants than in *S. bulbocastanum*. In greenhouse experiments, all of the transgenic *S. tuberosum* plants exhibited a low level of susceptibility and the pathogen was able to sporulate to a small degree (Fig. 1).

***RB*-transgenic tuber resistance assays.** The results of wounded tuber inoculations indicated that tubers from transgenic lines are as susceptible to *P. infestans* as the

nontransgenic controls (Fig. 2). A slight increase in susceptibility to *P. infestans* in SP951 tubers was observed when compared with nontransformed Katahdin controls ($P = 0.008$). However, this result was

not consistent among the transgenic lines. Although the lesion sizes of inoculated SP2423 and SP2564 tubers were slightly larger than their respective Superior and Dark Red Norland tubers, this increase

Fig. 2. *RB* gene did not increase resistance to *Phytophthora infestans* in tubers. Three tubers from each *Solanum tuberosum* cultivar and the corresponding transgenic lines were inoculated with zoospores of *P. infestans*. The lesion volume was calculated from the width and depth of each lesion 10 days after inoculation.

Fig. 1. Late blight resistance of potato cultivars transformed with the *RB* gene from *Solanum bulbocastanum*. **A**, Nontransformed Katahdin and **B**, *RB*-transgenic SP951 plants were spray inoculated with the US-8 940480 strain of *Phytophthora infestans* at a concentration of 75,000 sporangia/ml. Photographs were taken 10 days after greenhouse inoculation. Some growth and sporulation of *P. infestans* (indicated by red arrow) was evident on *RB*-transgenic lines.

was not statistically significant. Russet Burbank and SP2105 tubers exhibited few disease symptoms.

Yield determination of *RB*-transgenic cultivars. ANOVA between replicated plots showed no significant effect of location within the test fields (blocks) for either 2005 or 2006. However, we did observe a significant year effect ($P < 0.0001$) on yield as well as a significant interaction between year and genotype ($P = 0.0003$). No significant differences in yield of US#1 or B-grade tubers were observed between Katahdin and the *RB*-transgenic SP951 (Fig. 3). In 2005, the average total yield of nontransgenic Katahdin was 1.9 kg per five-plant plot and the yield of SP951 was 1.8 kg/plot whereas, in 2006, the yields of each were 7.8 kg/plot. Although the difference was not statistically significant, nontransgenic Russet Burbank yielded slightly less than its *RB*-transgenic counterpart SP2105 in both years. The total average yields of nontransgenic Russet Burbank were 0.5 and 2.5 kg/plot in 2005 and 2006, respectively, whereas the average yields of SP2105 were 0.9 and 3.2 kg/plot. Both transgenic Superior (SP2423) and transgenic Dark Red Norland (SP2564) yielded slightly less than the nontransgenic plants, although the reduced yields were not statistically significant according to *t* tests ($P > 0.05$). The total yields of nontransgenic Superior and SP2423 were 1.1 and 1.3 kg/plot, respectively, in 2005 and 6.2 and 5.0 kg/plot in 2006. The yields of Dark

Red Norland and SP2564 were 0.7 and 0.9 kg/plot in 2005 and 7.7 and 6.8 kg/plot in 2006, respectively. Overall, the combined yield of *RB*-containing transgenic lines was not significantly different from their nontransgenic counterparts ($P = 0.708$).

DISCUSSION

Late blight remains one of the most serious and costly diseases of cultivated potato, and major efforts are being made to identify novel late-blight-resistance genes in wild species. The incorporation of these genes into cultivated potato is a critical step in the process of developing resistant cultivars. The creation of stable transgenic potato plants that contain an *R* gene from another potato species is relatively simple and has only a minor impact on the genetics of the recipient cultivated plant, which has undergone years of selection for quality production traits. The effect of the *RB* gene within various *S. tuberosum* cultivars consistently resulted in high levels of resistance in the lines used in these experiments. All of the *RB* transgenic lines exhibited an increase in resistance to *P. infestans* when compared with their nontransgenic counterparts. The increase in foliar resistance did not significantly decrease tuber yield, the most important aspect of potato production value, in the absence of late blight. We also were unable to detect any significant effect on yield after the incorporation of the *RB* gene into various potato cultivars.

Breeding for resistance to late blight in potato historically has been aimed at foliar interactions between the plant and pathogen. Tuber resistance is also of great importance because infection of tubers can lead to total loss of the harvest during storage or render a crop unsuitable for seed production. Genetic evidence suggests that some resistance genes in potato have the ability to confer both foliar and tuber resistance (15,16,20). Other results indicate the contrary, where there is no correlation between foliar and tuber resistance (10,21). Recently, it has been suggested that any correlation between resistances is dependent on the gene(s) involved (15). In the results of experiments presented here, introduction of the *RB* gene to *S. tuberosum*, which confers strong resistance to foliar late blight infection, did not confer tuber resistance in transgenic plants. This loss of resistance in tubers is not due to tissue-specific transcription of the *RB* transgene because tubers of *RB*-transgenic plants contain *RB* mRNAs (J. Bradeen, personal communication). Therefore, the loss of the resistance phenotype likely is due to instability of the *RB* protein or an ability of *P. infestans* to circumvent *RB*-mediated resistance during the tuber infection process.

S. bulbocastanum accession PT29, from which the *RB* gene was cloned, as well as several somatic hybrids between PT29 and *S. tuberosum* consistently display a high level of foliar resistance to *P. infestans*

Fig. 3. Comparison of yield in potato cultivars transformed and not transformed with the *RB* transgene for resistance to late blight. Three replicates of five-hill plots were planted with *RB*-transgenic Katahdin (SP951), Russet Burbank (SP2105), Superior (SP2423), and Dark Red Norland (SP2564) as well as nontransgenic controls in **A**, 2005 and **B**, 2006. Tubers were hand graded and divided into US#1 (dark gray bars) and B (light gray bars) grades. Data shown are the mean yields of each plot. There were no significant differences between the yield of the transgenic and control potato plants according to *t* tests at $P = 0.05$.

(8,14). The broad-spectrum nature of *RB* resistance suggests an ability to recognize an effector that is either essential to *P. infestans* virulence or is well conserved among all isolates. Regardless, *RB* is expected to be a valuable addition to resistance breeding programs because it could permit a significant reduction in the amount of fungicide required to prevent an outbreak of late blight. Encouragingly, the introduction of *RB* into breeding lines should have little or no impact on total yield.

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