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Correlation Between Transcript Abundance of the *RB* Gene and the Level of the *RB*-Mediated Late Blight Resistance in Potato

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Numerous disease-resistance genes have been cloned and characterized in various plant species. Only a few of these reported genes were transcriptionally induced or had enhanced transcription upon pathogen infection. Here, we report that transcription of the *RB* gene, which was cloned from the wild potato species *Solanum bulbocastanum* and confers resistance to potato late blight, was significantly increased after inoculation with the late blight pathogen *Phytophthora infestans*. Different *RB* transgenic lines showed different levels of resistance, which were correlated with the amounts of *RB* transcript in the transgenic plants. Different transgenic lines also showed different patterns of *RB* transcription 1, 3, and 5 days after *P. infestans* inoculation. Interestingly, the *RB* gene showed a higher basal level of transcription and a more dramatic transcriptional increase upon inoculation in *S. bulbocastanum* than in all potato transgenic lines. Our results revealed a predictive correlation between transcript abundance of the *RB* gene and the level of the *RB*-mediated late blight resistance. High level of resistance was associated with a combination of rapid *RB* transcript induction immediately after pathogen infection followed by the steady production of *RB* transcript. Thus, the transcription level of the *RB* gene provides a valuable marker for selecting and deploying *RB*-containing potato lines for late blight control.

Disease resistances in wild species are among the most important traits targeted by plant breeders in germplasm and cultivar development. Numerous resistance genes have been transferred from wild species into almost every major crop. Although most of these resistance genes introgressed from wild species were short-lived due to dynamic changes in the corresponding pathogen populations, some genes have provided effective and durable resistance for many years in agriculture. For example, the *Mi* gene of tomato, which was transferred from *Lycoperisicon peruvianum*, has provided effective resistance against several root-nematode species since the 1940s (Sorribas et al. 2005; Williamson 1998). The stem rust resistance gene *Sr26* in wheat was transferred from *Agropyron elongatum* (Knott 1968). *Sr26* is still effective and provides important and durable stem rust resistance for Australian wheat varieties (Dundas et al. 2004; Mago et al. 2005).

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Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating potato disease worldwide (Duncan 1999; Garelik 2002). Control of late blight in the United States relies almost exclusively on fungicide application. Annual late blight costs to United States growers were estimated to be $288 million, or approximately $500 per hectare (Guenthner et al. 2001). Due to the constant genetic shifts of the *P. infestans* populations and the explosive nature of disease development, late blight can cause a complete loss of the crop (Fry 2008). At least 11 race-specific late blight resistance genes, all from *Solanum demissum,* have been incorporated into numerous potato varieties (Black et al. 1953; Malcolmson and Black 1966). However, all of these genes have already been overcome by specific *P. infestans* strains, although some of these resistance (*R*) genes still provide some level of resistance in certain regions. Interestingly, two late blight resistance genes discovered in the Mexican diploid species *Solanum bulbocastanum*, *RB* (*Rpi-blb1*) and *Rpi-blb2*, confer broad-spectrum resistance against various *P. infestans* strains (Song et al. 2003; van der Vossen et al. 2003, 2005). Discoveries of these two genes have provided hopes that these genes may be more "durable" than the classic race-specific *R* genes previously used in potato breeding.

Transgenic potato lines containing the *RB* gene showed a high level of resistance in both greenhouse and field tests (Halterman et al. 2008; Kuhl et al. 2007; Song et al. 2003). We noticed that transgenic lines developed from the same potato cultivar often showed different levels of resistance. The speed of lesion development varied significantly among different lines. Such differences were particularly noticeable when a high concentration of sporangia was applied during greenhouse inoculation tests. In order to better understand what factors may influence the resistance of different transgenic lines, we characterized a set of transgenic lines that were developed from susceptible potato (*Solanum tuberosum*) variety Katahdin, each containing different numbers of the *RB* gene. We found that the transgenic lines with multiple copies of the *RB* gene consistently showed higher levels of resistance than the lines containing a single copy of the *RB* gene. In addition, a correlation between the basal amount of *RB* transcript and the copy number of the *RB* gene was found among the transgenic lines. Bradeen and associates (2009) has independently discovered a similar correlation between *RB* transcript abundance and the level of *RB*-mediated resistance. Our results showed that the amount of *RB* transcript, especially during the first 24 h after *P. infestans* infection, is a critical factor for determining the level of *RB*-mediated late blight resistance.

RESULTS

Copy numbers of the *RB* gene in transgenic potato lines.

'Katahdin', a potato cultivar highly susceptible to late blight, was transformed using the cloned *RB* gene (Song et al. 2003). The *RB* gene construct pCLD04541 includes the putative native promoter located within 2,365 bp of sequence upstream from the 5′ untranslated region (UTR) as well as 2,196 bp downstream of the 3′ UTR. Southern blot hybridization was used to estimate the copy numbers of 28 independent transgenic lines. A 4,573-bp polymerase chain reaction (PCR) fragment, which spans the neomycin phosphotransferase II (*NPTII*) gene within the left border of pCLD04541 (Fig. 1), was used as a probe. This 4,573-bp sequence does not contain *Eco*RI or *Hin*dIII sites. Thus, a single hybridization band would indicate that the transgenic line contains a single *RB* gene insertion. Most of the 28 lines contained one to two insertions of the *RB* gene, but transgenic lines containing up to four copies of the *RB* gene were also found (Fig. 1). A representative sample of transgenic lines with *RB* copy numbers ranging from one to four were chosen for further investigation.

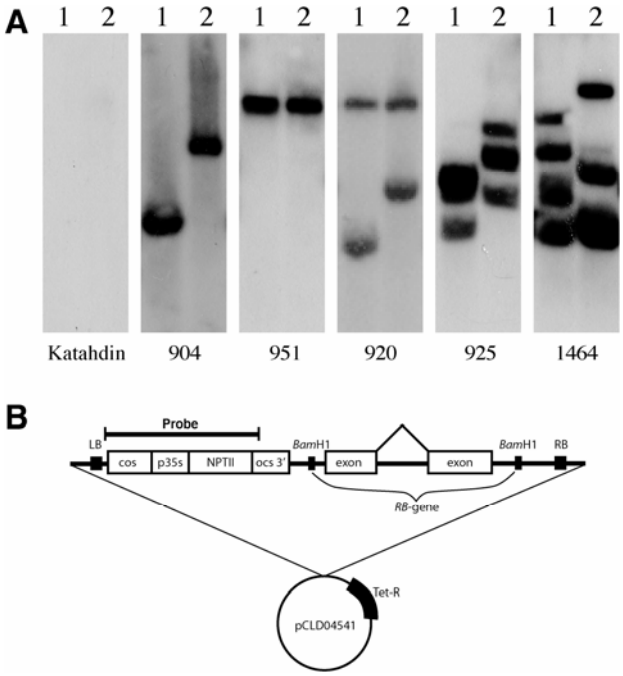

Fig. 1. Southern blot hybridization analysis of the *RB* gene copy number in the transgenic 'Katahdin' lines. **A,** Genomic DNA was digested with *Eco*RI (lanes 1) and *Hin*dIII (lanes 2), respectively. Transgenic 'Katahdin' lines 904, 951, 920, 925, and 1464 were estimated to contain 1, 1, 2, 3, and 4 copies of the *RB* gene, respectively. **B,** The probe used in Southern blot hybridization spans the cos site, 35s promoter (p35s), and the neomycin phosphotransferase II gene (NPTII) as well as the beginning of the terminator (ocs 3′) within the left border (LB) of the pCLD04541 plasmid.

The level of resistance to late blight is correlated with the copy number of the *RB* gene in the transgenic lines.

We repeatedly noticed that different transgenic lines exhibit different levels of lesion formation and sporulation in greenhouse late blight inoculation tests. The differences among the transgenic lines were particularly obvious when the plants were inoculated with a high concentration of sporangia (>100,000 sporangia/ml). A transgenic 'Katahdin' line, 1464, containing four copies of the *RB* gene (Fig. 1) consistently showed a high level of resistance during inoculation tests.

In order to better understand the putative correlation between the resistance level and the copy number of the *RB* gene in the transgenic lines, we chose 11 transgenic 'Katahdin' lines (Table 1), representing six independent insertion events, for greenhouse late blight resistance evaluation. We performed four different inoculation tests using two different isolates of *P. infestans*. Different concentrations of the sporangia inoculums were applied in the tests. We scored whole plants for visible signs of resistance 4, 7, and 10 days after inoculation (dai) (Fig. 2; Table 1). At 4 dai, we observed a marginal increase in disease symptoms for the genotypes that contain one copy of the *RB* gene (lines 904 and 951) as well as the susceptible control 'Katahdin'. At 7 dai, there was a significant increase in disease severity in 'Katahdin' as well as lines 904 and 951. Lines with two to four copies of *RB* still held similar resistance scores (Supplementary Fig. 1). At 10 dai, we observed the largest differences across the genotypes. There were significant differences in disease development between those lines with one, two, and three copies of the *RB* gene. Transgenic lines with three and four copies of the *RB* gene as well as *S. bulbocastanum* accession PT29, from which the *RB* gene was originally cloned (Song et al. 2003), showed a similar level of resistance (Fig. 2). These results showed that the late blight resistance level in transgenic 'Katahdin' lines was correlated with the copy number of the *RB* transgene.

Table 1. Greenhouse late blight resistance scores for *RB*-transgenic lines 10 days postinoculation

Line	*RB* copy number	ME93 86,100 sporangia/ml[x]	BB 90,100 sporangia/ml[x]	BB 120,400 sporangia/ml[x]	ME93 130,555 sporangia/ml[x]
Katahdin	0	1.17 ± 0.41	0.67 ± 1.15	2.67 ± 0.94	2.50 ± 0.71
904	1[y]	3.67 ± 0.58	2.33 ± 0.58	4.50 ± 0.24	3.33 ± 0.00
905	1[y]	4.00 ± 1.00	3.00 ± 1.00	3.83 ± 0.24	3.17 ± 0.24
1006	1	5.50 ± 0.71	3.00 ± 1.00	2.83 ± 0.24	4.33 ± 0.47
951	1	6.00 ± 1.00	4.33 ± 0.58	5.50 ± 0.24	4.67 ± 0.00
918	2[y]	5.67 ± 0.58	5.33 ± 0.58	6.00 ± 0.00	4.17 ± 0.24
920	2[y]	6.67 ± 0.58	7.00 ± 1.00	5.00 ± 0.47	6.17 ± 0.24
922	2[y]	6.67 ± 0.58	5.33 ± 1.53	4.83 ± 0.24	6.50 ± 0.24
898	2	6.00 ± 1.00	5.00 ± 1.73	4.17 ± 0.71	4.83 ± 0.24
925	3[y]	7.33 ± 0.58	7.00 ± 0.00	7.33 ± 0.47	6.50 ± 0.24
926	3[y]	7.67 ± 0.58	7.67 ± 0.58	7.33 ± 0.00	7.17 ± 0.71
1464	4	7.67 ± 0.58	6.00 ± 1.73	6.67 ± 0.47	7.17 ± 0.24
PT29[z]	…	8.00 ± 0.00	8.00 ± 0.00	8.00 ± 0.00	8.00 ± 0.00

[x] Values shown are averages ± standard deviation.
[y] Southern blot results reveal the exact same banding pattern in those lines with the same copy number of the *RB* gene, indicating that these lines were derived from the same transgenic events.
[z] *Solanum bulbocastanum* accession PT29.

Quantitative evaluation of late blight resistance of the transgenic lines.

In order to further verify the different levels of resistance observed among the *RB*-transgenic lines, a green fluorescent protein (GFP)-tagged strain, *P. infestans* 208m2, was used to quantify the pathogen infection, as previously described by Bhaskar and associates (2008). Five transgenic plants with one to four copies of the *RB* gene were investigated using this method. Photographing the fluorescing GFP-*Phytophthora* infected cells allowed us to measure the area of pathogen growth across the leaf surface, represented as particle counts using ImageJ analysis software (Fig. 3). A higher particle count indicates greater spread of the pathogen across a leaf. Images were taken from inoculated leaf samples 5 dai.

The data from the quantitative fluorescence imaging were highly correlated with the greenhouse resistance evaluation (Pearson correlation $P < 0.05$, average $R^2 = 0.72$). There was no significant difference between the particle counts within different inoculation spots on the same leaves (sub-sampling factor, $P = 0.8263$), but there was a significant difference between the particle counts from different transgenic lines ($P < 0.001$). Fishers least significant difference (LSD) tests performed in SAS (alpha of 0.05) indicated that the susceptible control 'Katahdin' had a significantly higher particle count followed by 904 and 951, each of which contains one copy of the *RB* gene (Fig. 3). The transgenic lines with two, three, and four copies of *RB* were not significantly different from each other but were significantly different from the genotypes without or with one copy of the *RB* gene (Fig. 3).

Transcript abundance of the *RB* gene in different transgenic lines.

Quantitative real-time (qRT)-PCR analysis was used to investigate a possible relationship between the amount of *RB* transcript and the number of *RB* genes in the different transgenic lines. The expression of the alpha tubulin gene was used to normalize the relative abundance of the *RB* transcript within each line. The amount of the *RB* transcript in different lines was then compared with the *RB* transcript level in the *RB*-'Katahdin' line 904, which contains one copy of the *RB* gene (Fig. 4).

We first compared the transcription of the *RB* gene in different lines without late blight inoculation. RNA was isolated from plants after 1, 2, and 3 weeks of growth in greenhouses. An interesting increase in basal expression levels was observed in plants that have multiple copies of the *RB* gene compared with line 904 (Fig. 4A and B). The same expression pattern was also observed when the plants were exposed to misting water for 24 h (Fig. 4D). Basal *RB*-transcript levels were significantly correlated with the *RB*-copy number (Pearson correlation $P < 0.01$, $R^2 = 0.97$).

A dynamic change of the *RB* gene expression was observed when the plants were challenged by *P. infestans*. One day after inoculation, line 920 (two copies of the *RB*) showed the highest level of transcription (Fig. 4E). At 3 dai, all transgenic lines showed the same level of *RB* transcription (Fig. 4F). At 5 dai however, the transgenic plants with multiple copies of the *RB* gene again showed a higher level of transcription than those with a single copy of *RB* (Fig. 4G), similar to that in the plants before inoculation.

Interestingly, the *RB* gene showed, on average, a 5.5-fold higher basal expression level of the *RB* gene (Fig. 4A through D) in *S. bulbocastanum* accession PT29. One day after inoculation, the *RB* gene showed a dramatic 37-fold higher level of expression in PT29 than line 904 (Fig. 4E). PT29 maintained a higher level of expression than all other transgenic lines 3 and 5 dai (Fig. 4F and G).

The *RB* gene expression data was also analyzed within each transgenic line over time and during inoculation, using week 1 as the relative comparison (Fig. 5). The expression of the *RB* gene peaked at 3 dai in lines with only one copy of the *RB* gene (Fig. 5A and B). In contrast, the *RB* transcription in transgenic lines 920 and 925 (two and three copies of the *RB*, respectively) peaked 1 dai, similar to the pattern in PT29 (Fig. 5C, D, and F). Line 1464 (four copies of *RB*) showed a slow induction of expression, peaking 5 dai (Fig. 5E).

Transcription of the *Sgt1* and *PR-5* genes in the transgenic lines.

The different and dynamic expression pattern of the *RB* gene in the transgenic lines prompted us to investigate the expression patterns of other defense-related genes, using the same RNA samples. The *Sgt1* gene is essential to many plant disease-resistance pathways, including the *RB*-mediated late blight resistance pathway (Bhaskar et al. 2008). The transcription level

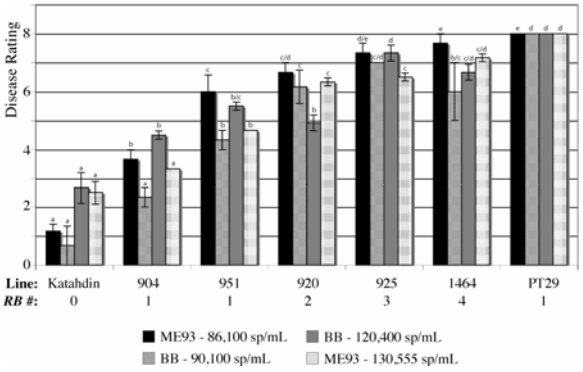

Fig. 2. Late blight resistance scores of the transgenic 'Katahdin' lines 10 days after late blight inoculation. Two different *P. infestans* strains (ME93 and BB) were used at four different inoculum concentrations. Different letters within each inoculation experiment represent significant differences (Fisher's least significant difference, alpha 0.05) between each *RB*-transgenic line. For example, the letter "e" within the ME93 86,100 sporangia per milliliter experiment (black columns) indicates that the resistance scores between lines 925, 1464, and PT29 are not significantly different.

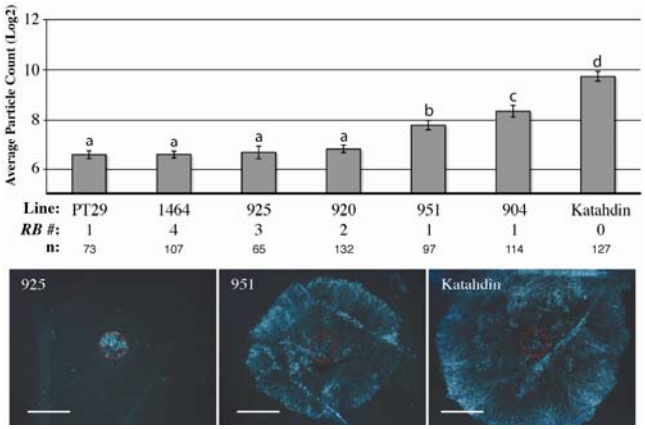

Fig. 3. Late blight resistance evaluation using a GFP-tagged *Phytophthora infestans* strain. **Upper,** Quantification of fluorescent sporangia and mycelium lesion growth. The *y* axis indicates the $\log_2$-transformed particle counts, which correspond to the fluorescent sporangia. n = the total number of lesions analyzed. The same letters indicate no significant differences between particle counts of the different genotypes, using Fisher's least significant difference (alpha 0.05). **Lower,** Representative images of green fluorescent protein–tagged *P. infestans* growth 5 days postinoculation. Bars represent 5 mm, and the red circle indicates the original location of the 10-µl inoculation drop. The fluorescent signals were restricted within the original inoculation spot in line 925 but spread out significantly in line 951 and 'Katahdin'.

of the *Sgt1* gene was similar among all transgenic lines before and 1 day after inoculation (Fig. 6A through E). A few exceptions showing increased transcription of *Sgt1* were observed in a few lines 3 and 5 dai (Fig. 6F and G), although the relative level of change was low compared with the changes of the *RB* gene. The *Sgt1* gene showed on average a 1.2-fold increase in expression one day after inoculation and maintained this level through 5 dai (Supplementary Fig. 2).

We also investigated the expression of pathogenesis-related gene 5 (*PR5*), which can be strongly induced by *P. infestans* inoculation (Wang et al. 2006). A similar level of *PR5* expression was observed in all lines (Supplementary Fig. 3). The susceptible control 'Katahdin' showed a seven- and 14.5-fold increase in expression of *PR5* 3 and 5 dai. The significant accumulation of *PR5* transcript was observed in all lines 3 dai (Supplementary Fig. 4).

DISCUSSION

Regulation of *RB* gene expression.

Although a large number of plant disease-resistance genes have been cloned, there has been very limited research on the

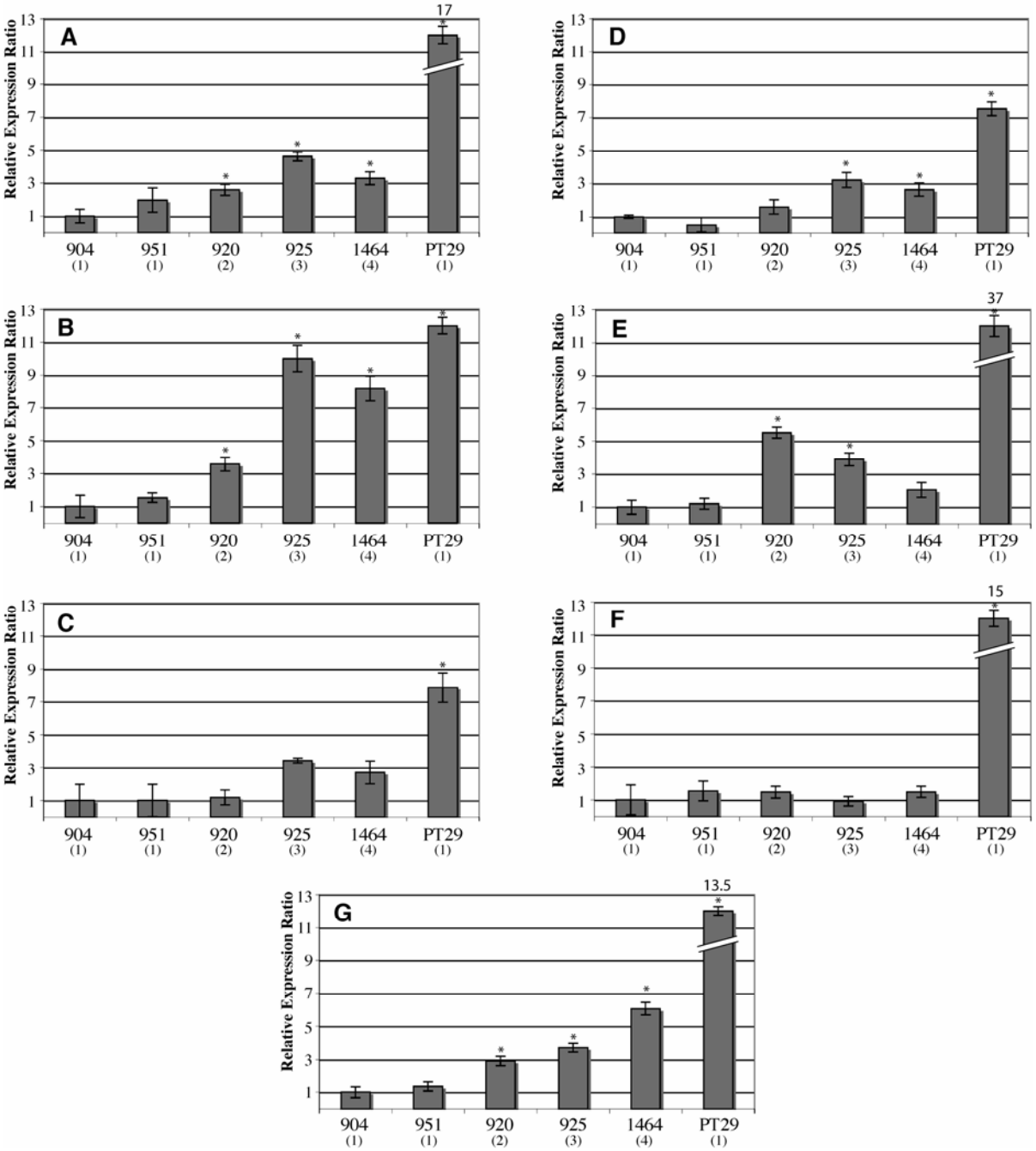

Fig. 4. Quantitative real-time polymerase chain reaction analysis of *RB* gene transcription in transgenic 'Katahdin' lines. The expression level of the *RB* gene in each genotype is relative to the expression level of line 904, which was used as the baseline expression value for all comparisons. The average relative expression ratios for three biological replicates are shown on the *y* axis. The numbers in parentheses indicate the copy number of the *RB* gene in each line. **A,** *RB* expression after 1 week of growth; **B,** *RB* expression after 2 weeks of growth; **C,** *RB* expression after 3 weeks of growth; **D**, *RB* expression after 24 h in the mist chamber, at approximately 4 weeks of growth; **E,** *RB* expression 1 day postinoculation with the *Phytophthora infestans*; **F,** *RB* expression after 3 days postinoculation; **G,** *RB* expression after 5 days postinoculation. Stars indicate samples that are significantly different from line 904 using a *t*-test (alpha = 0.05) for the delta cycle threshold values.

regulation of plant *R* gene expression. Most *R* genes showed a low basal level of expression (Ayliffe et al. 1999; Huang et al. 2005; Shen et al. 2002; Tan et al. 2007). These genes, such as the potato late blight *R* gene *R3a*, did not increase their expression levels after pathogen challenge (Huang et al. 2005). However, the expression of some disease resistance genes was either induced only after pathogen infection (Gu et al. 2005; Romer et al. 2007; Yoshimura et al. 1998) or was significantly enhanced upon pathogen infection (Cai et al. 1997; Halterman et al. 2003; Levy et al. 2004; Radwan et al. 2005). The expression of some *R* genes can also be influenced by the host genetic background (Cao et al. 2007) and by other environmental factors that may be favorable to pathogen infection (Wang et al. 2001).

The *RB* gene is constitutively transcribed in all transgenic lines as well as in *S. bulbocastanum* (Fig. 4). The PT29 accession of *S. bulbocastanum* contains only a single copy of *RB* (Song et al. 2003). Yet, the expression level of the *RB* gene is significantly higher in *S. bulbocastanum* than in all the transgenic lines (Fig. 4). These results show that the genetic background (*S. bulbocastanum* or *S. tuberosum* cultivar Katahdin) influences the expression of the *RB* gene. Polyploidy can impact the expression of many genes in potato. However, the changes of gene expression due to ploidy alteration are usually within the twofold level (Stupar et al. 2007). Thus, the seven- to 17-fold difference in *RB* expression between *S. bulbocastanum* and *RB*-'Katahdin' cannot be attributed to the ploidy difference. Most strikingly, *RB* expression in *S. bulbocastanum* increased 37-fold higher than *RB*-'Katahdin' line 904 one day after *P. infestans* infection (Fig. 4E), suggesting that the genetic background can affect the basal expression as well as the inducibility of *RB* transcription. Bradeen and associates (2009) observed different average resistance levels of the *RB* transgenic lines developed from different potato cultivars, which also substantiates the impact of genetic background on *RB*-mediated resistance.

Pepper gene *Bs3* confers resistance to bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*. *Bs3* is transcriptionally activated by the *X. campestris* pv. *vesicatoria* effector AvrBs3. Interestingly, the recognition specificity resides within the *Bs3* promoter and is determined by the binding of AvrBs3 to the defined promoter region (Romer et al. 2007). Similarly, the *Xa27*-mediated bacterial blight resistance in rice was also determined by the activation of the *Xa27* gene upon pathogen infection and the activation specificity was attributable to the *Xa27* promoter (Gu et al. 2005). *RB* appears to be regulated by a different mechanism, i.e., *P. infestans* infection does not activate but enhances the expression of the *RB* gene. *RB* expres-

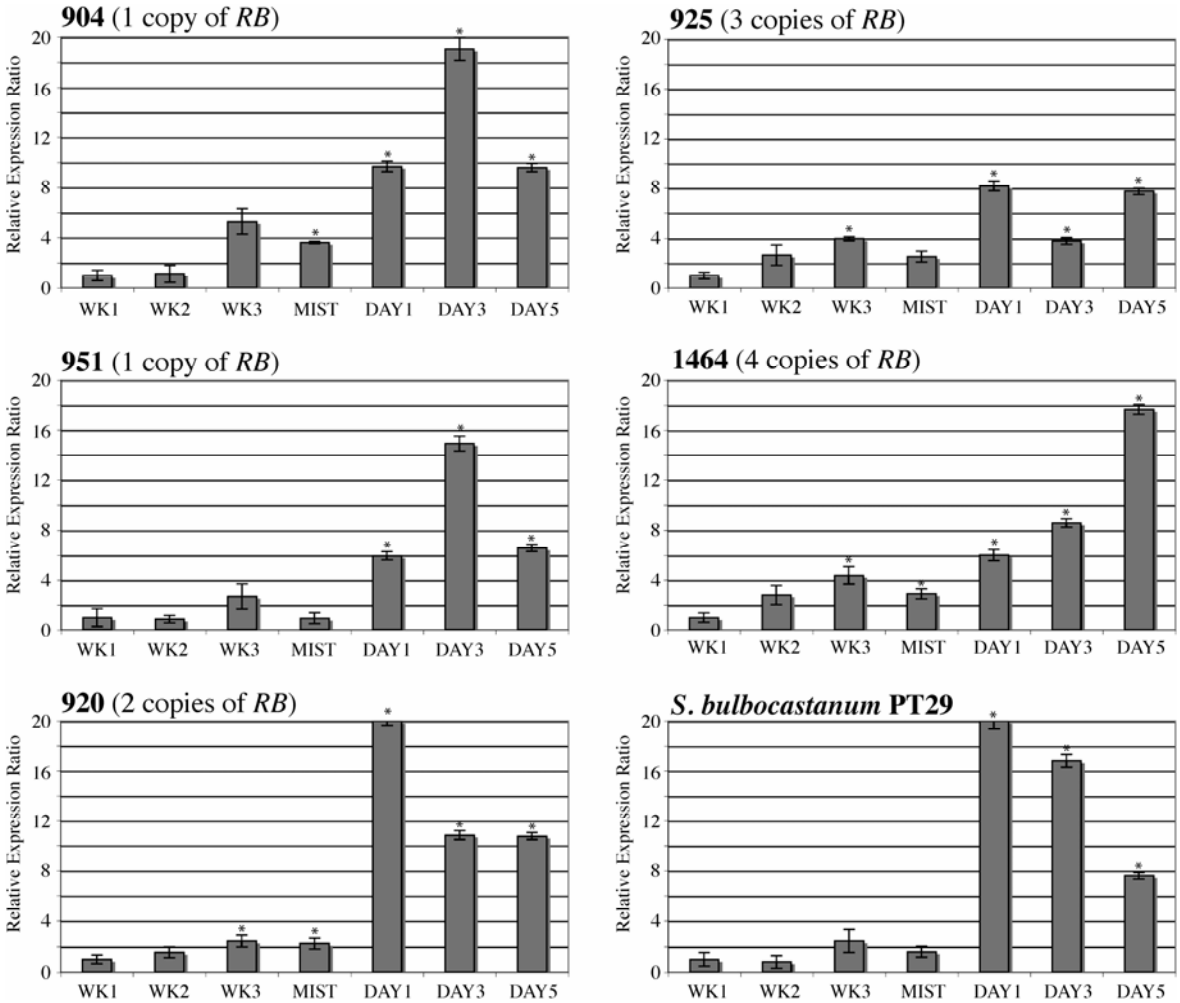

Fig. 5. *RB* gene expression under different conditions in transgenic 'Katahdin' lines. The average relative expression ratios for three biological replicates are shown on the *y* axis. Leaf samples were collected at the timepoints shown on the *x* axis, and week 1 is the baseline expression value against which all other timepoints were compared. Timepoints are as follows: after one week of growth (WK1), after two weeks of growth (WK2), after three weeks of growth (WK3), 24 h in the mist chamber (MIST), 1 day postinoculation with *Phytophthora infestans* (DAY1), 3 days postinoculation (DAY3), and 5 days postinoculation (DAY5). Stars indicate samples that are significantly different from the week 1 baseline expression value using a *t*-test (alpha = 0.05) for the delta cycle threshold values.

sion was not changed by mock inoculations (Fig. 5) or by wounding (data not shown), confirming the *P. infestans*–mediated enhancement of *RB* transcription. It will be interesting to further investigate whether the native promoter of the *RB* gene plays a role in the *P. infestans*–mediated transcription enhancement and whether plasmid pCLD04541 includes all the *cis* elements that are required for *RB* gene induction and transcription.

Relationship between the level of disease resistance and the transcript abundance of the corresponding resistance gene.

Transcription of some *R* genes is significantly enhanced upon pathogen infection, suggesting that the amount of the *R* gene product may be important for the resistance mediated by such genes. A well-studied case on the relationship between *R* gene transcript abundance and the level of resistance is the bacterial blight resistance gene *Xa3* in rice. The *Xa3* gene displays different resistance levels and spectrums in *indica* and *japonica* rice, two major subspecies of Asian cultivated rice (Cao et al. 2007). These differences are correlated with different levels of expression of the *Xa3* gene in the two subspecies. In addition, overexpression of *Xa3* with a constitutive strong promoter enhanced the resistance in both subspecies, whereas expression of *Xa3* driven by a pathogen-induced weak promoter impaired resistance (Cao et al. 2007).

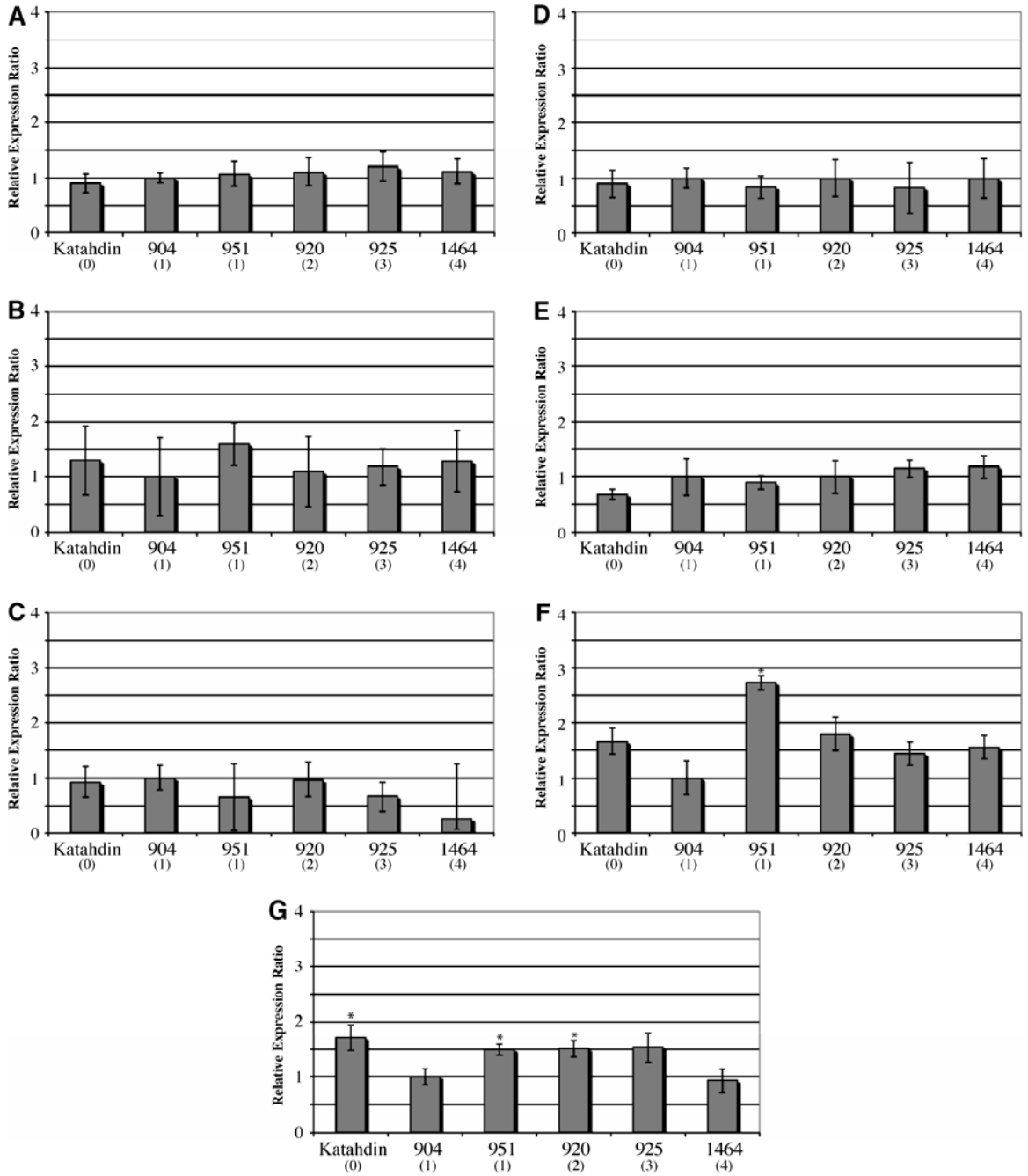

Fig. 6. Expression of the *Sgt1* gene in transgenic 'Katahdin' lines. The average relative expression ratios for three biological replicates are shown on the *y* axis. The expression level of the *Sgt1* gene in each genotype is relative to the expression level of line 904, which was used as the baseline expression value for all comparisons. The numbers in parentheses indicate the copy number of the *RB* gene in each line. **A,** After 1 week of growth; **B,** After 2 weeks of growth; **C,** After 3 weeks of growth; **D,** After 24 h in the mist chamber, at approximately 4 weeks of growth; **E,** at 1 day postinoculation with the green fluorescent protein–*Phytophthora infestans* isolate; **F,** at 3 days postinoculation; **G,** at 5 days postinoculation. Stars indicate samples that are significantly different from line 904 using a *t*-test (alpha = 0.05) for the delta cycle threshold values.

The different levels of late blight resistance of various *RB*-'Katahdin' lines have been verified by both greenhouse inoculation tests and field resistance evaluations (Bradeen et al. 2009). The *RB*-'Katahdin' lines displaying higher levels of resistance in both the greenhouse and field tests contained multiple copies of the *RB* gene and produced higher basal amounts of the *RB* transcript (Fig. 4). Interestingly, tubers from plants containing the *RB* gene did not show resistance to late blight (Halterman et al. 2008), which is potentially related to a relatively lower amount of transcription of the *RB* gene in the tubers or potential instability of RB proteins in the tubers (Halterman et al. 2008).

The peak timing of *RB* transcription appears to be one factor related to the level of resistance. One day after *P. infestans* inoculation, all transgenic lines with multiple copies of *RB* produced more *RB* transcripts than lines with only one copy of *RB*. *S. bulbocastanum* accession PT29 produced a striking 37-fold more *RB* transcript than *RB*-transgenic line 904 (one copy of *RB*) 1 dai (Fig. 4E). At 3 dai however, all transgenic lines produced a similar amount of *RB* transcript (Fig. 4F). Transgenic lines 904 and 951 (one copy of *RB*) appeared to have a relatively slow induction of *RB* expression and the transcription peaked at day 3 after inoculation (Fig. 5), while the *RB* transcription in lines 920 (two copies of *RB*), 925 (three copies of *RB*), and PT29 peaked at day 1 after inoculation (Fig. 5). The first 24 h after inoculation may be critical for prohibiting or retarding the growth of the pathogen. The presence of significantly more *RB* transcripts at this early stage possibly plays a critical role in the higher levels of resistance.

Transgenic line 1464 (four copies of *RB*) showed an interesting pattern of *RB* transcription. At 1 dai, line 1464 had not accumulated as much *RB* transcript as lines 920 and 925. However, line 1464 showed a steady increase in *RB* gene expression over the first 5 days of infection. Thus, the combination of rapid induction immediately upon pathogen infection followed by the steady production of the *RB* transcript in later stages, which was clearly displayed in PT29, may be the key to higher levels of *RB*-mediated resistance.

The *Sgt1* and *PR5* genes also showed an increase in expression after *P. infestans* inoculation. However, the transcription changes of these two genes were similar among transgenic lines with different copy numbers of the *RB* gene (Fig. 6). These results support the idea that transcripts from the *RB* gene rather than from genes in the resistance signaling pathway have a direct impact on the *RB*-mediated resistance.

Strategy for deployment of the *RB* gene.

The broad-spectrum resistance conferred by the *RB* gene makes it an extremely valuable resource for late blight resistance breeding. Both transgenic and nontransgenic approaches are available to deploy the *RB* gene (Colton et al. 2006; Halterman et al. 2008; Kuhl et al. 2007). Since the levels of *RB*-mediated resistance are correlated with the transcript abundance of the *RB* gene at both the basal level and after *P. infestans* inoculation, the transcription of the *RB* gene can potentially be used as a criterion in selection of *RB*-containing transgenic lines or breeding lines. PT29 shows a significantly higher level of basal expression of the *RB* gene. Thus, potato may be able to tolerate high levels of *RB* gene expression. The *RB* gene and the *Xa3* gene in rice share similar characteristics of an association between resistance and the genetic background and transcript abundance. Interestingly, overexpression of the *Xa3* gene in rice also showed no remarkable morphologic and developmental differences from wild type (Cao et al. 2007). Thus, the breeding value of the *RB* gene can be fully exploited by selecting and deploying potato lines with a high basal level of *RB* expression.

MATERIALS AND METHODS

Plant materials and growth.

Late blight susceptible cultivar Katahdin was transformed with pCLD04541, which contains the full-length *RB* gene and the native promoter (Song et al. 2003). All transgenic lines used in this study were maintained as in vitro plantlets. For the evaluation of resistance in greenhouses and the quantification of GFP-*P. infestans*, plantlets were transplanted into 4-in. pots filled with standard potting media. Plants were grown in a completely randomized design for 5 weeks and were randomly reoriented once a week to minimize variation. The greenhouse was set for 18 h of daylight, a daytime temperature between 17 and 19°C, and a nighttime temperature between 13 and 15°C.

Southern blot hybridization.

DNA was isolated from leaf tissue and was quantified using a Nanodrop (ThermoFisher Scientific, Hampton, NH, U.S.A.). Approximately 20 μg of DNA from each line was digested with *Eco*R1 or *Hin*dIII and was blotted on a membrane. The blots were probed with a 4,573-bp PCR fragment, which spans the *NPTII* gene within the left border of the plasmid pCLD04541. This DNA fragment was amplified from pCLD04541 using forward primer 5′GCGGACGGCCAATAC TCAAC and reverse primer 5′CCCTCATATCAACTACTACG. The probe was generated from 100 ng of the PCR product using the DNA StripEZ kit (Ambion/Applied Biosystems, Austin, TX, U.S.A.). The blot was probed overnight at 65°C using standard protocols (Sambrook and Russell 2001).

Greenhouse late blight resistance evaluation.

The *RB*-'Katahdin' lines together with 'Katahdin' and *S. bulbocastanum* accession PT29 were evaluated for resistance to *P. infestans* in an environmentally controlled mist chamber at the University of Wisconsin-Madison using published procedures (Colton et al. 2006). Eleven lines were tested with two *P. infestans* isolates, ME93 (US930287, A2 mating type with avirulence [*Avr*] genes 8 and 9) and BB (US940480, A2 mating type with *Avr* genes 8 and 9). Four different inoculation tests were performed using the following sporangial concentrations: BB (90,100 and 120,400 sporangia per milliliter) and ME93 (86,100 and 130,555 sporangia per milliliter) in order to represent high and low disease pressure. Measurements of the late blight infection were scored according to the Malcolmson scale (Cruickshank et al. 1982). The scale was based on percentage of foliage infected, and scores were as follows: 9 = no visible infection, 8 = <10% infection, 7 = 11 to 25%, 6 = 26 to 40%, 5 = 41 to 60%, 4 = 61 to 70%, 3 = 71 to 80%, 2 = 81 to 90%, 1 = >90%, and 0 = 100% infection. An average score and standard deviation for the resistance of each clone was determined using three replicates in each experiment. Statistical analysis was performed using SAS 9.1.3 for Windows (SAS Institute Inc., Cary, NC, U.S.A.). Plants with a score of 7 or higher on day 10 are considered resistant.

GFP-*P. infestans* inoculation and quantification.

The *RB*-'Katahdin' lines together with 'Katahdin' and PT29 were inoculated by the GFP-tagged *P. infestans* isolate 208m2 provided by F. Mauch (University of Friberg, Switzerland) (Si-Ammour et al. 2003). Three complete biological replicates were performed with at least two replicates of each transgenic plant. The average sporangial count for each biological replicate was 86,666, 165,037, and 124,629 sporangia per milliliter. The area of *P. infestans* growth was quantified as described previously (Bhaskar et al. 2008) with only a minor modification; the "watershed particle analysis" function was used instead of the "particle analysis." This tool can better distinguish large

masses of mycelium or sporangia. The resulting data are recorded as particle counts, which correspond to the total number of fluorescing sporangia on the leaf surface. A higher particle count indicated that a larger area had been taken over by the growing *P. infestans*. Data quality tests and assessments of the particle count data indicated that a $\log_2$ transformation was necessary to satisfy the assumption of normality. All data analysis, analysis of variance, and LSD was performed using SAS 9.1.3 for Windows.

Quantification of *RB* gene transcription.

Three biological replicates of selected *RB*-'Katahdin' lines together with 'Katahdin' and *S. bulbocastanum* accession PT29 were used to quantify *RB* transcripts, using a total of eight plants per line in each replicate. Transcription quantification was conducted for the alpha tubulin gene, the *Sgt1* gene, and the *PR5* gene (Supplementary Fig. 5). Leaf tissue was collected 1, 2, and 3 weeks after the tissue culture plants were transplanted in greenhouses. Tissue was also collected 1 week after the plants were placed in the mist chamber before inoculation. Experiments were performed in the mist chamber using the GFP-*P. infestans* isolate 208m2 with average sporangial concentrations of 124,629 and 91,111 sporangia per milliliter. Tissue was then collected 1, 3, and 5 dai. One leaf was removed from the top third of each transgenic plant within one biological replicate at one of the sampling points and was used for RNA isolation.

RNA was isolated using the Qiagen plant RNeasy kit using the on-column DNase digestion according to the manufacturer's instructions (Qiagen, Valencia, CA, U.S.A.). RNA was then treated with Turbo DNA-Free (Ambion/Applied Biosystems) and was quantified using Ribogreen (Invitrogen, Carlsbad, CA, U.S.A.). Super Script III reverse transcriptase and random primers were used to generate the cDNA (Invitrogen). Controls without reverse transcriptase were tested for all cDNA reactions. cDNAs were treated with RNase (Promega, Madison, WI, U.S.A.) and were cleaned using the QIAquick PCR purification kit (Qiagen). cDNA was diluted 1:5 in distilled water and was used for qRT-PCR along with DYNAMO SYBR Green master mix (Finnzymes, New England Biolabs, Ipswich, MA, U.S.A.) and 10 nM primers. qRT-PCR was performed in triplicate for each sample on the MJ Research Opticon 2 (Biorad, Hercules, CA, U.S.A.), using the primers and corresponding annealing temperature listed in Table 2. The following protocol was performed for all qRT-PCR experiments: 15 min at 95°C, 40 cycles of 20 s at 94°C, 20 s at the corresponding annealing temperature, 30 s at 72°C, followed by a plate read, and then, a melting curve of 50 to 95°C with 0.2 degree steps, hold 2 s, followed by a final extension step of 10 min at 72°C.

Alpha tubulin and *Elongation Factor 1* (*ef1*) were previously identified as the best candidates for reference genes when performing qRT-PCR with RNA from potato inoculated with *P. infestans* (Nicot et al. 2005). Both genes were tested using the cDNA from all three biological replicates from the first 2 weeks of growth, in order to determine which was suitable for use as the normalizing reference gene. The *alpha tubulin* gene demonstrated the most consistent cycle threshold (Ct) values and was used for further analysis (Supplementary Fig. 6). Primers were also designed for the *Sgt1* and *PR5* genes (Table 2). All primer efficiencies were calculated by comparing the delta Ct values with the log input RNA amount using template dilutions; the absolute value of the slopes were less than 0.1. The primer efficiencies were similar between the diploid and tetraploid genomes and the variance minimal. We therefore assumed that the total tubulin mRNA output per transcriptome is similar between the diploid and tetraploid genomes analyzed. All statistical analysis was performed using the delta Ct values. *T*-tests with unequal variance against the baseline (*RB*-'Katahdin' line 904 or week 1 [WK1]) were performed in the R statistical analysis environment (Yuan et al. 2006). The comparative Ct method (delta delta Ct method) (Schmittgen and Livak 2008) was used to generate the fold change results, using either *RB*-'Katahdin' line 904 or the WK1 samples as the baseline. Positive and negative correlations were tested using Pearson's product moment correlation coefficient.

Table 2. Primers used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis

qRT-PCR primer	Sequence	°C[z]	Product size (bp)
RB-1	F: CACGAGTGCCCTTTTCTGAC	50	213
RB-1′	R: ACAATTGAATTTTTAGACTT		
50-alpha-tubulin-A	F: AAGTTTGATCTGATGTATGC	50	270
50-alpha-tubulin-a	R: GAAGACCACACCAAGTAATA		
Sgt1-A	F: TAGGGCTAAAGAGGCGTTCA	57	159
Sgt1-a	R: TTCGCATCAACAACAGCTTC		
PR5-A	F: GCATAAGAGATTACGACACC	57	136
PR5-a	R: TCCACCCAACACTTTAGC		
57-alpha-tubulin-A	F: AATTTGTCGACTGGTGTCCT	57	152
57-alpha-tubulin-a	R: GTCAATGCGAGAGAAGACCT		

[z] Melting temperature.

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LITERATURE CITED

Ayliffe, M. A., Frost, D. V., Finnegan, E. J., Lawrence, G. J., Anderson, P. A., and Ellis, J. G. 1999. Analysis of alternative transcripts of the flax L6 rust resistance gene. Plant J. 17:287-292.

Bhaskar, P. B., Raasch, J. A., Kramer, L. C., Neumann, P., Wielgus, S. M., Austin-Phillips, S., and Jiang, J. M. 2008. *Sgt1*, but not *Rar1*, is essential for the *RB*-mediated broad-spectrum resistance to potato late blight. BMC Plant Biol. 8:8.

Black, W., Mastenbroek, C., Wills, W. R., and Petersen, L. C. 1953. A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity of *Solanum demissum* derivatives. Euphytica 2:173-179.

Bradeen, J. M., Iorizzo, M., Mollov, D. S., Raasch, J., Kramer, L. C., Millett, B. P., Austin-Phillips, S., Jiang, J. M., and Carputo, D. 2009. Higher copy numbers of the potato *RB* transgene correspond to enhanced transcript and late blight resistance levels. Mol. Plant-Microbe Interact. 22:437-446.

Cai, D. G., Kleine, M., Kifle, S., Harloff, H. J., Sandal, N. N., Marcker, K. A., KleinLankhorst, R. M., Salentijn, E. M. J., Lange, W., Stiekema, W. J., Wyss, U., Grundler, F. M. W., and Jung, C. 1997. Positional cloning of a gene for nematode resistance in sugar beet. Science 275:832-834.

Cao, Y. L., Ding, X. H., Cai, M., Zhao, J., Lin, Y. J., Li, X. H., Xu, C. G., and Wang, S. P. 2007. Expression pattern of a rice disease resistance gene *Xa3*/*Xa26* is differentially regulated by the genetic backgrounds and developmental stages that influence its function. Genetics 177:523-533.

Colton, L. M., Groza, H. I., Wielgus, S. M., and Jiang, J. M. 2006. Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene *RB* derived from a wild potato species. Crop Sci. 46:589-594.

Cruickshank, G., Stewart, H. E., and Wastie, R. L. 1982. An illustrated assessment key for foliage blight of potatoes. Potato Res. 25:213-214.

Duncan, J. M. 1999. *Phytophthora*—An abiding threat to our crops. Microbiol. Today 26:114-116.

Dundas, I. S., Verlin, D. C., Park, R. F., Bariana, H. S., Anugrahwati, D. R., Shepherd, K. W., McIntosh, R. A., and Islam, A. K. M. R. 2004. Progress in development of new rust resistant wheat using chromosomes from uncultivated relatives. Pages 122-124 in: Proceedings of the 54th Australian Cereal Chemistry Conference and 11th Wheat Breeders

Assembly. C. K. Black, J. F. Panozzo, and G. J. Rebetzke, eds. Canberra, Australia.
Fry, W. 2008. *Phytophthora infestans*: The plant (and *R* gene) destroyer. Mol. Plant Pathol. 9:385-402.
Garelik, G. 2002. Taking the bite out of potato blight. Science 298:1702-1704.
Gu, K. Y., Yang, B., Tian, D. S., Wu, L. F., Wang, D. J., Sreekala, C., Yang, F., Chu, Z. Q., Wang, G. L., White, F. F., and Yin, Z. C. 2005. *R* gene expression induced by a type-III effector triggers disease resistance in rice. Nature 435:1122-1125.
Guenthner, J. F., Michael, K. C., and Nolte, P. 2001. The economic impact of potato late blight on US growers. Potato Res. 44:121-125.
Halterman, D. A., Wei, F. S., and Wise, R. P. 2003. Powdery mildew-induced *Mla* mRNAs are alternatively spliced and contain multiple upstream open reading frames. Plant Physiol. 131:558-567.
Halterman, D. A., Kramer, L. C., Wielgus, S., and Jiang, J. M. 2008. Performance of transgenic potato containing the late blight resistance gene *RB*. Plant Dis. 92:339-343.
Huang, S. W., Van der Vossen, E., Kuang, H., Vleeshouwers, V. G., Zhang, N., Borm, T. J. A., van Eck, H. J., Baker, B., Jacobsen, E., and Visser, R. G. F. 2005. Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. Plant J. 42:251-261.
Knott, D. R. 1968. Translocations involving *Triticum* chromosomes and *Agropyron* chromosomes carrying rust resistance. Can. J. Genet. Cytol. 10:695-696.
Kuhl, J. C., Zarka, K., Coombs, J., Kirk, W. W., and Douches, D. S. 2007. Late blight resistance of *RB* transgenic potato lines. J. Am. Soc. Hort. Sci. 132:783-789.
Levy, M., Edelbaum, O., and Sela, I. 2004. Tobacco mosaic virus regulates the expression of its own resistance gene *N*. Plant Physiol. 135:2392-2397.
Mago, R., Bariana, H. S., Dundas, I. S., Spielmeyer, W., Lawrence, G. J., Pryor, A. J., and Ellis, J. G. 2005. Development of PCR markers for the selection of wheat stem rust resistance genes Sr24 and Sr26 in diverse wheat germplasm. Theor. Appl. Genet. 111:496-504.
Malcolmson, J. F., and Black, W. 1966. New R genes in *Solanum demissum* and their complementary races of *Phytophthora infestans*. Euphytica 15:199-203.
Nicot, N., Hausman, J. F., Hoffmann, L., and Evers, D. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J. Exp. Bot. 56:2907-2914.
Radwan, O., Mouzeyar, S., Nicolas, P., and Bouzidi, M. F. 2005. Induction of a sunflower CC-NBS-LRR resistance gene analogue during incompatible interaction with Plasmopara halstedii. J. Exp. Bot. 56:567-575.
Romer, P., Hahn, S., Jordan, T., Strauss, T., Bonas, U., and Lahaye, T. 2007. Plant pathogen recognition mediated by promoter activation of the pepper *Bs3* resistance gene. Science 318:645-648.
Sambrook, J., and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
Schmittgen, T. D., and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nature Protoc. 3:1101-1108.
Shen, K. A., Chin, D. B., Arroyo-Garcia, R., Ochoa, O. E., Lavelle, D. O., Wroblewski, T., Meyers, B. C., and Michelmore, R. W. 2002. *Dm3* is one member of a large constitutively expressed family of nucleotide binding site–leucine-rich repeat encoding genes. Mol. Plant-Microbe Interact. 15:251-261.
Si-Ammour, A., Mauch-Mani, B., and Mauch, F. 2003. Quantification of induced resistance against *Phytophthora* species expressing GFP as a vital marker: Beta-aminobutyric acid but not BTH protects potato and *Arabidopsis* from infection. Mol. Plant Pathol. 4:237-248.
Song, J., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberlach, G. T., Liu, J., Kuang, H., Austin-Phillips, S., Buell, C. R., Helgeson, J. P., and Jiang, J. M. 2003. Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc. Natl. Acad. Sci. U.S.A. 100:9128-9133.
Sorribas, F. J., Ornat, C., Verdejo-Lucas, S., Galeano, M., and Valero, J. 2005. Effectiveness and profitability of the Mi-resistant tomatoes to control root-knot nematodes. Euro. J. Plant Pathol. 111:29-38.
Stupar, R. M., Bhaskar, P. B., Yandell, B. S., Rensink, W. A., Hart, A. L., Ouyang, S., Veilleux, R. E., Busse, J. S., Erhardt, R. J., Buell, C. R., and Jiang, J. M. 2007. Phenotypic and transcriptomic changes associated with potato autopolyploidization. Genetics 176:2055-2067.
Tan, X. P., Meyers, B. C., Kozik, A., Al West, M., Morgante, M., St Clair, D. A., Bent, A. F., and Michelmore, R. W. 2007. Global expression analysis of nucleotide binding site-leucine rich repeat-encoding and related genes in Arabidopsis. BMC Plant Biol. 7:20.
van der Vossen, E., Sikkema, A., Hekkert, B. L., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., and Allefs, S. 2003. An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J. 36:867-882.
van der Vossen, E. A. G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A., and Allefs, S. 2005. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant J. 44:208-222.
Wang, X. B., El Hadrami, A., Adam, L. R., and Daayf, F. 2006. Local and distal gene expression of *pr-1* and *pr-5* in potato leaves inoculated with isolates from the old (US-1) and the new (US-8) genotypes of *Phytophthora infestans* (Mont.) de Bary. Environ. Exp. Bot. 57:70-79.
Wang, Z. X., Yamanouchi, U., Katayose, Y., Sasaki, T., and Yano, M. 2001. Expression of the Pib rice-blast-resistance gene family is up-regulated by environmental conditions favouring infection and by chemical signals that trigger secondary plant defences. Plant Mol. Biol. 47:653-661.
Williamson, V. M. 1998. Root-knot nematode resistance genes in tomato and their potential for future use. Annu. Rev. Phytopathol. 36:277-293.
Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z. X., Kono, I., Kurata, N., Yano, M., Iwata, N., and Sasaki, T. 1998. Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. Proc. Nat. Acad. Sci. U.S.A. 95:1663-1668.
Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N. 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7:85.