

Resistance to Potato Leafroll Virus in Potato Plants Transformed with the Coat Protein Gene or with Vector Control Constructs

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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ABSTRACT

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The results of greenhouse evaluations of potato leafroll virus (PLRV) resistance in transgenic potato plants are reported. Russet Burbank and Ranger Russet plants were transformed via *Agrobacterium*-mediated gene transfer with three constructs, one carrying the native viral coat protein (CP) gene, another containing a modified form of the CP gene designed to optimize protein expression, and a third consisting of the binary vector plasmid only. A statistically significant correlation ($P < 0.01$) was found between incidence of infection within replications of a given clone and virus titers of the infected plants. Resistance to PLRV titer buildup as

measured by enzyme-linked immunosorbent assay was observed among clones in all three construct populations. Plants transformed with the modified CP gene did not exhibit greater levels of resistance to PLRV than plants transformed with the native CP gene. Of particular interest are several highly resistant lines that were derived by transforming Ranger Russet with the control construct carrying only vector sequences and the neomycin phosphotransferase (NPTII) gene. This resistance, which is heritable and has been verified in three separate inoculations, indicates that a component of PLRV resistance in transgenic potato is associated with vector DNA or the tissue culture process. Possible explanations for this novel type of resistance and its implications are discussed.

Additional keyword: somaclonal variation.

Potato leafroll luteovirus (PLRV) is an aphid-transmitted, phloem-limited virus that causes severe yield losses in potato worldwide. Current control measures are limited to use of clean seed, control of the green peach aphid vector, and planting when aphid populations are naturally low. Transgenic resistance may offer an attractive alternative means of reducing PLRV damage. Coat protein (CP)-mediated resistance has been documented for several plant-virus combinations (23) and has been found to delay virus accumulation and symptom expression (1). The mechanism of CP-mediated resistance has yet to be elucidated, but at least in some cases CP production is required (26) and the level of resistance achieved has been reported to be proportional to the amount of transgenic CP produced (10,12).

The PLRV CP gene, which encodes a 23 kDa protein, is located in the 3' region of the genome (31) and contains an internal open reading frame (ORF) encoding a 17 kDa (17K) putative single-stranded nucleic acid-binding protein (33). Translation initiation at the AUG of the internal protein has been found to exceed that of the CP gene by a factor of 7 (32). Potato plants transformed with native PLRV CP gene constructs have been found in our and other laboratories (4,15,34) to accumulate lower titers of PLRV upon infection, but little or no transgenic CP has been detected in these plants. One possible explanation is interference of the preferentially expressed internal 17K protein with translation of the CP gene. To test this possibility, and in an attempt to design a more effective construct, we designed a modified CP

gene in which the two potential internal initiation ATGs of the 17K ORF were mutated to ACGs, thus destroying the 17K ORF while conserving the amino acids that are in the reading frame for CP. In addition, the -3 position was mutated from a T to A. This change should increase translational initiation of the CP ORF (18). In order to compensate for the variability associated with transgenic plants, several independently derived transformants of each construct were tested along with a large number of clones transformed with the vector containing only the neomycin phosphotransferase (NPTII) gene but no viral sequences.

MATERIALS AND METHODS

Construction of pPLRV4-612. The CP gene was cloned from pPLRV4-323-1, which contains the coding sequence bordered by upstream and downstream viral regions (O. P. Smith, *unpublished sequencing data*). The gene was isolated as a *Cfr* 10I-*AccI* fragment and after treatment with the Klenow fragment of DNA Pol I was blunt-end ligated into the *SmaI* site of pGEM-7Zf(+) (Promega Corporation, Madison, WI). Orientation of the gene (31) was confirmed by digestion with *Bam*HI (data not shown). This intermediate construct contained the 627-bp CP coding sequence bordered by 5' and 3' viral sequences of 54 bp and 3 bp, respectively, and included the internal 17K ORF (31). To place the gene under transcriptional control of the duplicated cauliflower mosaic virus 35S promoter (17) and the tml terminator (21), the gene was cloned into pCGN1431 (Calgene Inc., *unpublished plasmid*) as an *Asp* 718-*Sst*I fragment. Finally, the gene with control signals was cloned as a 3.1-kbp *Pst*I fragment into the binary vector pCGN1547 (21) to produce pPLRV4-612 for the *Agrobacterium*-mediated transformation of potato (Fig. 1).

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Transcriptional orientation of the gene in pPLRV4-612 was parallel to NPTII, which was confirmed by digestion with *Hind*III (data not shown).

Construction of pPLRV4-622. The 5' region of the CP gene was engineered to increase protein translation by truncation of the nontranslated leader sequence to the 40 base pairs of the CP subgenomic RNA (31,32), mutation of a pyrimidine (T) to a purine (A) at the -3 position relative to the ATG initiator codon (18), and removal of two translational start sites for the internal 17K ORF by mutating two initiator codons (ATG) to ACG. These modifications were produced by the mutual priming

and cloning of two partially complementary oligonucleotides (Fig. 2) as described by Ausubel et al (3). The 0.2-kbp extension product was cloned into pGEM-7Zf(+) as an *Asp* 718-*Hind*III fragment and gene assembly was completed by additional cloning of the 0.5-kbp *Nar*I-*Hind*III fragment from pPLRV4-412. After verification by DNA sequence analysis (28), the modified gene was sequentially cloned into pCGN1431 and pCGN1547 as described above to produce pPLRV4-622 (Fig. 1).

Agrobacterium transformation. All three binary vector constructs were introduced into *Agrobacterium* strain LBA4404 by the freeze-thaw method. An *Agrobacterium* culture was grown overnight in liquid MG/L medium (5 g mannitol, 1 g glutamic acid, 0.25 g KH_2PO_4 , 0.1 g NaCl, 0.1 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1 μg biotin, 5 g tryptone, 2.5 g yeast extract per liter, pH 7.0) and adjusted to an OD_{600} between 1 and 1.5. Following a 10-min centrifugation at 3,000 g, the bacterial pellet was resuspended in 1 ml of ice-cold MG/L. Aliquots of 200 μl were frozen immediately at -70°C and either stored at -70°C for up to several months or used immediately. To transform the bacteria, 1 μg of purified plasmid DNA was added to a thawed 200- μl aliquot of competent cells and refrozen quickly by immersion in liquid nitrogen for 5 min. The mixture was then thawed quickly by placing the tube at 37°C for 5 min, 1.8 ml MG/L medium was added, and the bacteria incubated at 30°C with gentle shaking for 4 h. Following a 30 s centrifugation at 12,000 g, the cell pellet was resuspended in 100 μl MG/L and spread on MG/L agar plates containing 50 $\mu\text{g}/\text{ml}$ of gentamicin.

Plant tissue culture. Plant material for *Agrobacterium* transformation was grown axenically on shoot medium (MS medium containing 20 $\mu\text{g}/\text{L}$ NAA) (22) in test tubes and under a 12-h light cycle ($1-1.4 \mu\text{E sec}^{-1}\text{m}^{-2}$). Transformants were cultured on shoot medium in test tubes under continuous light provided by Gro-Lux fluorescent bulbs (GTE Corp., Danvers, MA).

Plant transformation. The altered CP sequence was introduced into three potato cultivars by co-cultivation with *Agrobacterium*. An *Agrobacterium* culture grown to an OD_{600} of 0.7 was washed twice with and resuspended in an equal volume of MS medium (22). Internodal sections of 5-10 mm length were cut from the stems of 4- to 6-wk-old axenically grown Ranger Russet or Russet Burbank plants and incubated for 5 min in the *Agrobacterium* suspension. Care was taken not to include any nodes with the stem pieces. The stem pieces were blotted dry with Whatman filter paper and placed on callus induction medium (6) containing 1 mg/L BAP in place of trans zeatin riboside and 30 g/L sucrose. After 2 days the stem pieces were transferred to 3C5ZR medium (29) containing 100 mg/L myo-inositol, 250 mg/L cefotaxime, and 50 mg/L kanamycin sulfate. The explants were transferred to fresh medium at weekly intervals. Callus formation commenced within 2 wk and most shoots were harvested between 9 and 12 wk

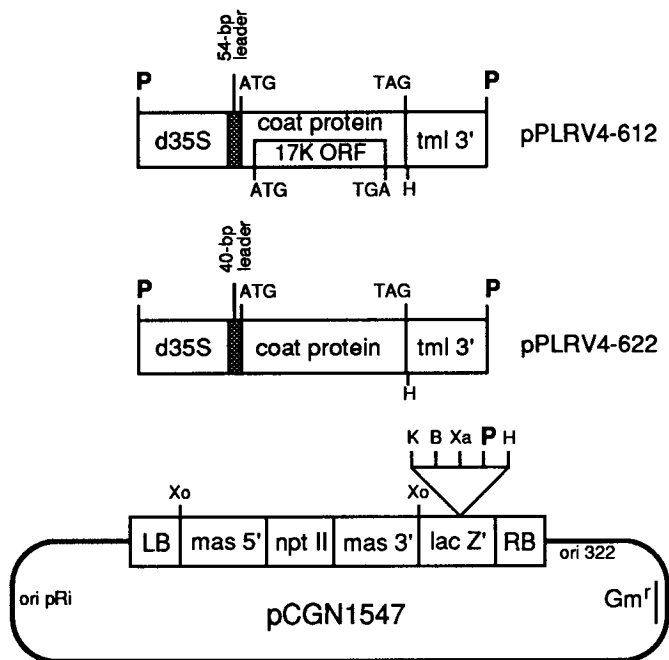


Fig. 1. Partial map of the cloning vector pCGN1547 and the virus constructs pPLRV4-612 and pPLRV4-622. The *Pst*I fragment of the modified coat protein gene was cloned into the *Pst*I site of the binary vector pCGN1547 to yield pPLRV4-612 and pPLRV4-622. The two *Xho*I sites of pCGN1547 are separated by 2,428 bp. Abbreviations used: d35S = duplicated cauliflower mosaic virus 35S promoter (17); 17K ORF = internal open reading frame encoding 17K single-stranded nucleic acid-binding protein (33); tml 3' = terminator of *tml* (21); LB = left T-DNA border; mas 5' = mannopine synthase promoter, npt II = neomycin phosphotransferase gene; mas 3' = mannopine synthase terminator; lacZ = β -galactosidase gene; RB = right T-DNA border. Restriction enzyme sites: B = *Bam*HI; H = *Hind*III; K = *Kpn*I; P = *Pst*I; Xa = *Xba*I; X0 = *Xho*I.

Oligonucleotide 1 (111 bases):

5'-CCCGGTACCG¹TTTACCTAAAGATTTCTCCACGTGCGATCAAT
TGA²TA[ATG]³AGTACGGTTCGTGGTTAAAGGAA[ACG]⁴TCA[ACG]⁵GT
GGTGTAACAACAACCAAGAAGGCGAAG-3'

Oligonucleotide 2 (110 bases):

5'-CCCAAGCTTCTTCGGCGCCTGGGTTGCCAGGGGCCGTGACCA
TAACCACTGGCTGAACCTCTGTTAGCGCGCCTGCGAAGGGATTGCC
TTCTTCGCCTTCTTGGTTGTTG-3'

Fig. 2. Sequences of the two partially complementary oligonucleotides used for construction of pPLRV4-622. Key nucleotides are identified with numbers: 1 = 5' end of the coat protein subgenomic RNA (32); 2 = native T mutated to A to place the coat protein ATG in an optimal translational context (18); 3 = translation initiation codon for the coat protein gene; 4, 5 = ATG of the internal 17K ORF mutated to ACG to block translational initiation.

after co-cultivation. Shoots of 5 mm length or longer were excised and placed onto shoot medium containing cefotaxime or ampicillin. All in vitro culture was at 21 C under continuous light provided by Gro-Lux fluorescent bulbs.

PCR analysis of transformants. Putative transformants that regenerated from kanamycin resistant callus were screened for the presence of the NPTII and CP gene using specific primers. Plant genomic DNA was isolated by freezing shoot tips of regenerants in liquid nitrogen and grinding the tissue to a fine powder using a minipestle in an Eppendorf tube. After addition of 566 μ l of extraction buffer (100 mM Tris 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM mercapto-ethanol and 1% sodium dodecyl sulfate [SDS]) the tubes were vortexed and incubated at 65 C for 10 min; then 160 μ l of 5 M KOAc was added to each tube, followed by vortexing and centrifugation for 15 min. The supernatant was removed and DNA precipitated with isopropanol. The pellet was washed twice in 70% EtOH and resuspended in 400 μ l of sterile water. One hundred nanograms of DNA was used for each polymerase chain reaction (PCR). PCR amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler 480 for 30 cycles at 94 C for 1 min, 55 C for 2 min, and 72 C for 2 min. Primers for the NPTII and CP gene coding regions were purchased from Genosys Biotechnologies (The Woodlands, Texas) and have the following sequences: NPTII Fwd - CTGATGCTCTTCGTC-AGATCA, NPTII Rev - CTGATGCTCTTCGTCAGATCA, PLRV Fwd - AGTACGGTCGTGGTTAAAGG, PLRV Rev - CTATTTGGGTTTTGCAAAG. The NPTII gene primers amplify a 414-bp fragment of the NPTII coding region and the PLRV primers amplify a 624-bp fragment of the PLRV CP coding region.

Southern analysis. Potato genomic DNA was isolated as described above, digested with *Xho*I and electrophoresed and blotted essentially as described by Brown et al (6). The coding regions of the NPTII and PLRV CP genes were radioactively labeled with [α ³²P]dATP by PCR and used as probes in the Southern hybridization analysis.

Northern analysis. Total plant RNA was isolated from approximately 100-mg batches of frozen leaf tissue as described (35). Glyoxylated RNA was electrophoresed in a 0.8% agarose gel and blotted onto Genescreen Plus (DuPont NEN Research Products, Boston, MA). Hybridizations were performed in a buffer containing 5% dextran sulfate, 1% SDS and 1 M NaCl and washes were performed in 2 \times SSC (1 \times SSC is 0.15 NaCl plus 0.015 M sodium citrate) and 1% SDS at 65 C.

Resistance testing. All plantlets tested for virus resistance originated from axenic culture. Plantlets included in a given test were subcultured as nodal cuttings at the same time. The nodal cuttings were allowed to root on sterile MS medium for 2–4 wk (time varied between experiments) prior to being transplanted into 3-in. plastic pots containing a soil mix (2:3:3:1 soil/mortar sand/peatmoss/medium vermiculite) that had been fumigated with methyl bromide at least 1 wk prior to use. Pots were covered with plastic bags and arranged in a randomized complete block design in a walk-in temperature-controlled incubator and exposed to either continuous light or 18 h light/6 h dark cycles. The plastic bags were removed after 5 days.

Viruliferous aphids, *Myzus persicae* (Sulz.), that had been raised on PLRV-infected *Datura* plants were kindly provided by Lee Fox (USDA, Yakima, WA). Potato plants were inoculated with PLRV by placing a *Datura* leaf piece holding 15–50 viruliferous aphids onto each potato plant at 7 or 14 days after transplanting. Within a few hours the aphids moved off the drying *Datura* leaf piece and onto the potato plantlet where they began feeding. The aphids were contained by covering each potato plantlet with an inverted plastic cup whose bottom had been replaced with nylon screen. After 4 days of feeding the aphids were killed by dipping the plants into a 1.92 ml/L aqueous solution of Pounce (permethrin, FMC Corp., Philadelphia, PA). Plants were then transferred to the greenhouse.

At various times after inoculation leaf tissue was analyzed for the presence and amount of virus. Five leaf disks, weighing on average 33 mg total, were harvested with a hole punch from the

apical leaves of each plant and ground with a Kontes pestle in an Eppendorf tube containing 0.5 ml of phosphate-buffered saline (PBS). One hundred microliters of this suspension was loaded into the wells of microtiter plates pre-coated with polyclonal antiserum to PLRV. After addition of 100 μ l of conjugate the plates were incubated overnight at 4 C. Plates were washed several times with PBS-Tween before addition of diethanolamine substrate. Absorbance at 450 nm was read and recorded at 1, 4, and 24 h after substrate addition using a Biotek EL-308 enzyme-linked immunosorbent assay (ELISA) plate reader connected to a Zenith 286 portable computer.

Each microtiter plate contained buffer controls, healthy tissue controls, and serial virus dilutions in sap of healthy plant tissue. Absorbance values obtained with virus standards were used to transform the absorbance values to virus concentration in leaf tissue. This allowed quantitation of virus concentrations over a wide range. For example, regression analysis of the twofold virus dilution standards used in inoculation 3 and ranging from 4–4,000 ng/g leaf tissue yielded a straight line that passed through the origin and had an *R* value of 0.997. Plants were classified as uninfected if their ELISA values after a 24-h incubation with substrate were not different from healthy control extracts.

Statistical analysis. Significant differences in virus titer between clones and populations of clones were determined by analyzing ELISA data converted to nanograms of virus per gram of leaf tissue. Analysis of variance was performed with Proc GLM (SAS) and least significant difference was used to rank and group clones tested in the same experiment. Populations of transgenic plants were compared using Proc GLM (SAS) with a nested design that reflected the design of the experiment (i.e., clone within construct within variety). Correlation coefficients of incidence of infection vs. log virus titer were determined using the statistics package of Microsoft Excel.

TABLE 1. Infection frequencies and average virus titers of Russet Burbank clones tested at 6 wk postinoculation^a

Clone	No. plants infected	Virus titer (ng/g) mean \pm SE	Population mean \pm SE
Modified coat protein gene construct			
622.26	4	1,850 \pm 780 a-c	2,830 \pm 930 a
622.33	6	1,990 \pm 590 a-c	
622.37	6	2,160 \pm 350 a-d	
622.90	6	3,020 \pm 720 a-e	
622.109	5	3,580 \pm 1,490 a-e	
622.116	6	4,390 \pm 640 c-f	
Native coat protein gene construct			
612.30.2	5	820 \pm 580 a	2,970 \pm 2,250 a
612.62.1	5	990 \pm 440 ab	
612.3.1	4	1,180 \pm 690 ab	
612.52.1	6	3,030 \pm 540 a-e	
612.1.4	4	5,010 \pm 2,020 d-f	
612.6.3	6	6,760 \pm 1,660 f	
Binary vector control construct			
1547.40.1	6	3,840 \pm 770 b-e	4,500 \pm 510 b
1547.25.1	5	4,560 \pm 1,360 c-f	
1547.16.1	6	5,090 \pm 1,660 ef	
Untransformed	6	9,700 \pm 890 g	9,700 \pm 890 c

^aSix plants of each clone were inoculated with potato leafroll virus and the titer mean was derived from all inoculated plants. There are large differences in virus titer among the independently derived transformants carrying the same construct. With the exception of the untransformed control plants, all of the clones tested here had tested positive for transgenic RNA expression in Northern blots. Grouping was by one-way analysis of variance. Clones sharing a letter are not significantly different from each other at the *P* < 0.05 level. Analysis of construct populations was by Student's *t* test (least significant difference) using General Linear Models Procedure (SAS). Construct populations sharing a letter do not differ significantly at the *P* \leq 0.06 level.

RESULTS

Statistically significant differences in virus resistance exist between transformants and control plants. In order to evaluate the degree of virus resistance conferred by each construct, the first inoculation was set up to include several independent transformants of each of the six construct-variety combinations. Thirty-three different clones were tested for virus resistance in six replications. Tables 1 and 2 list the means and standard errors obtained with each clone and the titer means of each construct population.

All of the transformants listed in Tables 1 and 2 have been shown via Northern blotting to produce transgenic CP mRNA or, in the case of vector control constructs, NPTII mRNA. All of the transformed Russet Burbank clones had significantly lower virus titers than the untransformed control (Table 1). Only four out of 15 transgenic Ranger Russet clones differed significantly from the untransformed control (Table 2). The lower number of statistically significant differences within Ranger Russet may be due to the lower virus titers observed in Ranger Russet combined with the larger standard error resulting from the larger number of uninfected plants.

Comparison of transformant populations. Analysis of variance shows no significant differences between the populations transformed with the two viral CP constructs in either Russet Burbank or Ranger Russet, indicating that the modifications made to the CP construct did not significantly increase its effectiveness. Also noteworthy is that the control construct population differs significantly from the untransformed control in Russet Burbank, but not in Ranger Russet. This may indicate an interaction between plant genotype and the control construct or the transformation process.

It is important to note that there are statistically significant differences in virus resistance among independently derived transformants of the same variety-construct combination, all of which are known to produce transgenic mRNA. Three out of the six construct-variety combinations contain clones that have significantly lower virus titers than other clones within the same category. Variation among individual clones transformed with a given construct is greater than the variation between the three groups of constructs (pPLRV4-612 vs. pPLRV4-622 vs. pCGN1547). It thus appears that the variability associated with generating an individual clone is larger than the effect of the construct.

Greenhouse test results are reproducible. A second inoculation with a larger number of replications was conducted to verify that

TABLE 2. Infection frequencies and average virus titers of Ranger Russet clones tested at 6 wk postinoculation²

Clone	No. plants infected	Virus titer (ng/g) mean ± SE	Population mean ± SE
Modified coat protein gene construct			
622.95	3	260 ± 190 a	2,360 ± 600 a
622.69	4	620 ± 310 a	
622.1	4	1,230 ± 630 ab	
622.5	4	4,180 ± 1,900 b-d	
622.91	6	5,420 ± 1,500 cd	
Native coat protein gene construct			
612.21.1	6	2,440 ± 690 a-c	3,690 ± 600 ab
612.6.4	5	2,510 ± 990 a-c	
612.9.1	4	2,850 ± 1,670 a-d	
612.28.2	6	4,910 ± 500 cd	
612.2.3	6	5,680 ± 1,570 cd	
Binary vector control construct			
1547.5.1	3	1,160 ± 720 ab	3,970 ± 640 ab
1547.2.1	6	3,310 ± 1,080 a-d	
1547.1.1	5	4,340 ± 1,320 b-d	
1547.9.1	5	4,960 ± 1,550 cd	
1547.4.1	5	5,990 ± 1,930 d	
Untransformed	5	4,640 ± 1,210 cd	4,640 ± 1,210 b

²See footnote to Table 1.

the PLRV resistance assay is reproducible and the titer differences of the first inoculation were meaningful. Table 3 shows a comparison of the average titer values of infected plants for all nine clones tested in both experiments. The Spearman rank correlation coefficient (r_s) of 0.88 indicates that the ranking of the clones by virus titer in the two experiments is not significantly different at $P < 0.002$ and thus that this greenhouse assay is reproducible. The correlation coefficient is also significant ($r_s = 0.767$, $P < 0.02$) when uninfected plants are included in the clonal means. The ranking of the clones that were included in a third inoculation was also found not to be significantly different from the first inoculation ($r_s = 0.733$, $P < 0.05$).

Some control construct transformants show extremely low virus titers. A third inoculation was performed, this time using up to 23 replications per clone, in order to more thoroughly compare the most resistant clones from the first two inoculations and to verify the high level of resistance that had been found unexpectedly among some of the vector control transformants. The ranked clones along with their mean titer values are listed in Table 4. CP construct transformants of Russet Burbank that had previously exhibited low virus accumulation continued to show high resistance. However, the most resistant Ranger Russet line was RR 1547.5.1, which had been transformed with a control construct only. The virus titer of this clone was near the detection limit of our ELISA assay and represented a 30-fold reduction in virus

TABLE 3. Reproducibility of greenhouse screen for potato leafroll virus resistance

Clone ^y	Inoculation 1	Inoculation 2
	Virus titer (ng/g) mean ± SE ^z	Virus titer (ng/g) mean ± SE ^z
RB 612.62.1	1,180 ± 480	930 ± 150 [20]
RB 612.6.3	6,760 ± 1,660	1,520 ± 300 [20]
RB 612.1.4	7,644 ± 1,800	2,490 ± 420 [19]
RB untransformed	9,700 ± 890	2,610 ± 430 [18]
RR 622.95	600 ± 240	210 ± 50 [17]
RR 622.69	940 ± 370	550 ± 190 [17]
RR 612.2.3	5,680 ± 1,570	870 ± 260 [16]
RR 612.28.2	4,910 ± 500	890 ± 240 [18]
RR untransformed	5,560 ± 980	1,120 ± 470 [15]

^yRB = Russet Burbank; RR = Ranger Russet.

^zAverage virus titers of all infected plants of clones tested in two different experiments are listed as ng/g fresh weight. Values for inoculations 1 and 2 were for 6 wk and 4 wk postinoculation, respectively. Six replicates of each clone were inoculated in the first inoculation. The number of replicates for each clone in the second inoculation is indicated in square brackets. The Spearman rank correlation test shows no significant difference in ranking order between the two inoculations at $P < 0.002$.

TABLE 4. Potato leafroll virus resistance in some of the most resistant transgenic plants²

Clone (no. plants inoculated)	Infection frequency (%)	Virus titer (ng/g) mean ± SE
RB 612.36.1 (22)	27	30 ± 30 a
RB 612.46.1 (22)	73	230 ± 60 ab
RB 612.1.4 (22)	77	460 ± 110 b
RB 612.30.2 (21)	82	550 ± 130 b
RB untransformed (22)	100	2,030 ± 180 c
RR 1547.5.1 (23)	30	10 ± 10 a
RR 622.95 (13)	54	100 ± 40 ab
RR 622.69 (13)	69	260 ± 100 ab
RR 1,547.2.1 (22)	64	260 ± 80 ab
RR 612.2.3 (22)	68	330 ± 90 b
RR untransformed (22)	86	710 ± 140 c

²Virus titers were measured 4 wk postinoculation and are given in ng virus per gram of leaf tissue. Clones sharing a letter are not significantly different from each other at the $P < 0.05$ level (one-way analysis of variance). Means are derived by averaging the titer values of all inoculated plants. The number of replications for each clone is given in parentheses after the clone number.

titer. The resistance exhibited by this clone is dominant and inherited simply (S. Nielsen-Preiss et al, *personal communication*). A second control transformant, RR 1547.2.1 also exhibited lower virus titers than several clones transformed with viral CP constructs and had significantly lower virus titer than the untransformed control (Table 4). In order to verify that these lines are not mislabeled transformants carrying the viral CP construct, they were subjected to PCR and Southern analysis. PCR analysis shows that although the NPTII gene amplifies correctly in both 1547 clones, neither produces the CP fragment (Fig. 3). Southern analysis confirms the absence of PLRV CP sequences in the plant transformed with vector DNA alone (Fig. 4): *Xho*I-digested genomic DNA probed with both the NPTII and CP gene shows that RR 1547.5.1 contains only the 2.4-kb internal NPTII fragment. The RR 622.95 clone that was used as a control shows at least two additional bands representing the CP construct that contains border fragments of two independent insertions. Both additional bands are larger than the minimum 3.6-kb region spanning the T-DNA from the *Xho*I site to the right border.

Virus titer and infection frequency are correlated. Virus resistance was manifested by reduced virus titers in infected plants rather than immunity to infection. However, clones showing reduced virus accumulation also showed a decrease in the proportion of infected individuals (Fig. 5). The correlation coefficient for incidence of infection vs. virus titer of infected plants is significant at $P < 0.01$ for each of the two cultivars separately and combined. To our knowledge this is the first time that such a correlation between virus titer in infected plants and infection frequency has been observed in transgenic plants.

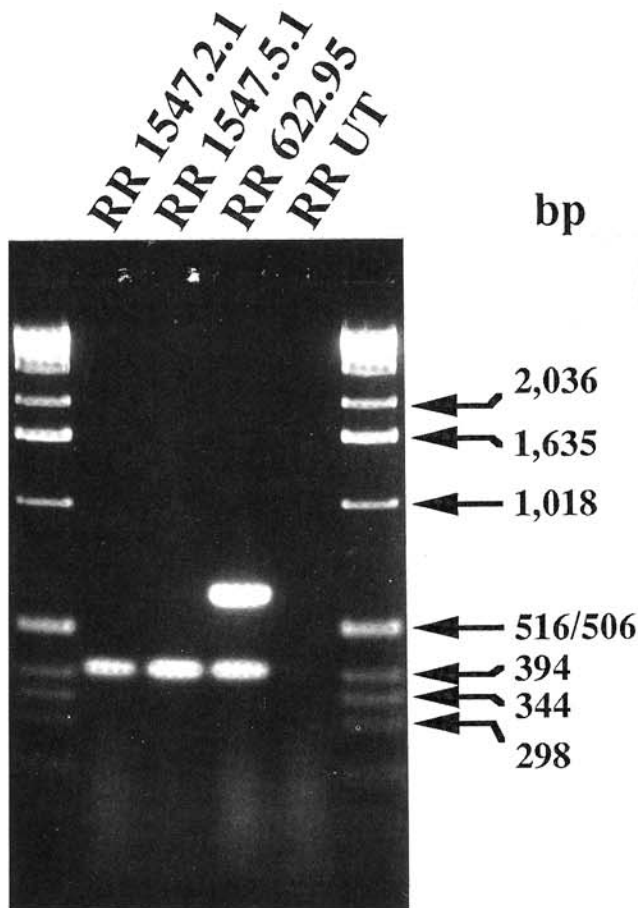


Fig. 3. Polymerase chain reaction amplification of plant genomic DNA shows no amplifiable coat protein sequences in vector control transformants. Plant genomic DNA was amplified in the presence of both NPT-II and coat protein-specific primers. All transformants exhibit the NPTII-specific amplification product (414 bp) but only the coat protein construct transformant RR 622.95 shows the coat protein-specific product (624 bp). The untransformed control plant shows neither amplification product.

Lower virus titer is reflected in plant phenotype. Throughout all inoculations we found that the virus titer as determined by ELISA was reflected by plant appearance. Plants with the highest virus titers generally exhibited severe potato leafroll symptoms consisting of stunting, curling, and yellowing of the upper leaves, as well as purple discoloration of the abaxial surface of young leaves. Plants with intermediate virus titers displayed these same symptoms to lesser degrees, whereas plants in which virus was not detected were green and healthy. This correlation between symptom expression and virus titer was observed in all lines. The high light intensity and warm temperatures in our greenhouse promoted symptom expression in infected plants.

DISCUSSION

Large variations in titer values were observed even among replications of the same genotype. This is not unusual and may be due to variable inoculation pressure or slight differences in

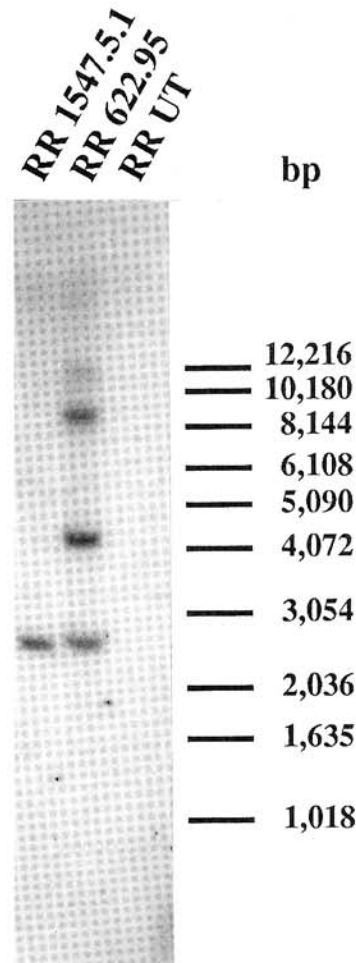


Fig. 4. Southern analysis of plant genomic DNA detects no coat protein coding sequence in RR1547.5.1. Ten micrograms of plant genomic DNA was digested with *Xho*I, run out on an agarose gel, blotted, and probed with the radioactively labeled coding regions of the NPTII gene and the coat protein gene. The control construct transformant RR 1547.5.1 shows only the NPTII-specific band (2.4 kb) and no additional sequences complementary to the coat protein region of the plasmid. The coat protein construct transformant RR 622.95 shows several additional bands corresponding to the *Xho*I fragment containing the PLRV coat protein gene and the right border. The additional bands are of variable size depending on the nearest genomic *Xho*I site, but all are greater than 3.6 kb, the expected minimum size from the internal *Xho*I site to the RB. The number of RB bands are indicative of at least two independent insertions in this transformant. As expected, the untransformed control plant (RR UT) shows no hybridization signal.

the physiology of transplants during or following inoculation. For example, in an early experiment differences were detected between replications subjected to different daylength regimes in the greenhouse. Many other as yet unrecognized environmental factors may influence titer by affecting plant growth and development.

Large variations in virus titer among replications of the same clone affect the statistical analysis by increasing the error term and the least significant difference between treatments. We found that we could have reduced the error and made the analysis more sensitive by excluding the occasional escapes in very susceptible clones from statistical analysis. However, in the more resistant lines the differentiation between escapes and resistant plants with extremely low titers becomes increasingly difficult. This combined with the fact that we found a correlation between the proportion of uninfected plants and the virus titer of the infected plants of a given clone (see Fig. 5) resulted in our decision to include all data in our statistical analysis.

Preliminary tests with our initial construct (pPLRV4-612) produced some transgenics with virus titer reductions similar to those observed by other workers (16,34). No transgenic CP was detected in these plants by Western analysis. We attempted to achieve higher levels of resistance by modifying the CP gene to more efficiently initiate translation at the CP initiation codon. Construct pPLRV4-622 contains an A at the -3 position and has the start codon of the highly expressed internal 17K protein altered to prevent internal initiation without changing the amino acid sequence of the CP. It also contains a shorter untranslated leader sequence. These alterations have not had the desired effect, as plants transformed with this construct still produced no detectable CP (data not shown), and the virus resistance did not differ significantly between the two constructs in either variety.

Both sense and antisense constructs of the PLRV CP gene have been shown by Kawchuk et al (16) to result in lower virus titers in transgenic potato. This, combined with the fact that threshold levels of transgenic CP are required for protection (7) and the lack of conclusive evidence for transgenically produced PLRV CP in potato, suggests that the virus resistance observed by us and others is not CP-mediated in the sense defined by Beachy (5) but may instead be mediated by the transgenic RNA.

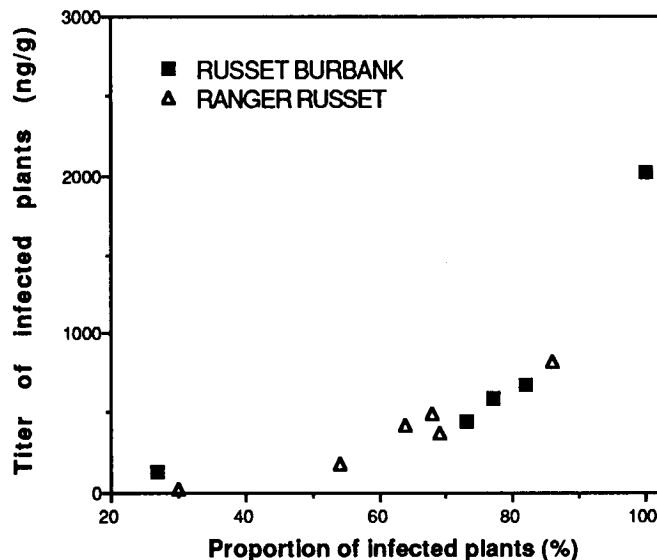


Fig. 5. Virus titer of infected plants vs. proportion of infected plants. Russet Burbank and Ranger Russet plants were inoculated as described in Materials and Methods and virus titers were assessed by enzyme-linked immunosorbent assay (ELISA) 4 wk postinoculation. Plants with ELISA values as low as healthy control plants 24 h after addition of substrate were judged to be uninfected. Correlation coefficients (r) of virus titer vs. incidence for Ranger Russet, Russet Burbank, and the combined total are 0.97 ($P < 0.002$), 0.97 ($P < 0.01$) and 0.94 ($P < 0.001$), respectively.

Tobacco plants with extreme resistance to tobacco etch virus have been produced by transformation with an untranslatable viral CP construct (19,20).

However, it is unclear how much of the effect observed with the CP constructs is due specifically to the CP coding sequence, since some of the most resistant plants were transformed with vector DNA alone. Only 50% of the plants transformed with CP sequences in either variety were significantly different from the least resistant control transformant and only 1/12 and none of the CP transformants of RB and RR, respectively, were significantly more resistant than the most resistant control transformant of that variety (Tables 1 and 2). It is thus reasonable to propose that we are not observing CP-mediated resistance but perhaps an RNA-mediated resistance. In this case, further attempts at engineering the CP gene to yield higher levels of protein may yet produce true CP-mediated resistance against PLRV in potato.

The attainment of very high levels of resistance with control constructs may indicate that transcription of any transgenic mRNA driven by a strong promoter can affect viral replication. In this case the transgenic mRNA levels in the phloem, or more specifically, the companion cells, are expected to be correlated with resistance. We were unable to detect a correlation between transgenic mRNA levels extracted from whole leaves and resistance to PLRV. For example, RR1547.4.1 and RR1547.5.1 contain similar levels of NPTII mRNA (data not shown) but differ significantly in their resistance response. However, this does not exclude a correlation at the phloem tissue level.

Another potential explanation for the reduced virus titers observed in some control construct transformants is that a gene contributing to susceptibility was fortuitously insertionally inactivated by the transgene. We are currently exploring this possibility by determining if the inserted DNA is genetically linked to the resistant phenotype.

Perhaps the most likely cause of the increased virus resistance observed in some of our control transformants is somaclonal variation. It can be speculated that the interaction between a phloem-limited virus and its host can be disrupted by a number of slight modifications to the plant growth pattern or physiology. Protoplast culture of potato is known to induce somaclonal variation (27) and has been proposed as a tool to generate novel phenotypes in potato (30). Even brief callus culture induces chromosome variation in potato (14,27) and Potter and Jones (25) found restriction fragment length polymorphisms for four single or low copy DNA probes in six out of 46 plants regenerated from leaf callus. Allicchio et al (2) found stable and unstable changes in isozyme expression patterns in potato plants regenerated from leaf callus. Evans et al (9) discovered statistically significant differences in several agronomic traits between controls and plants regenerated from leaf, rachis, or stem piece explants. Furthermore, Jongedijk et al (13) found that only 18% of transgenic Bintje potato plants transformed with potato virus X CP were true to type in all 50 morphological traits examined. This indicates that the act of transformation or the tissue culture period associated with transformation can produce significant variation among transformants. It would be interesting to determine the frequency and degree of PLRV resistance in Ranger Russet plants regenerated from calli resulting from mock transformation. It was recently shown by Hirochika (11) that protoplast culture and *Agrobacterium*-mediated leaf disk transformation of *N. tabacum* activates quiescent retrotransposons that may be one of the mechanisms of somaclonal variation. Since our transformation protocol is essentially identical to that used by Hirochika, we may assume that similar retrotransposons would have been activated in the course of our transformations, if they exist in the potato lines used in our experiments. Identification of such retrotransposons in potato may thus provide us with a tag for the genetic elements involved in susceptibility to PLRV.

The surprising observation that some control transformants are extremely resistant to PLRV infection, combined with the observation that transgenic effects are relatively small, points out the need to include equal numbers of control and test transformants in resistance tests involving transgenic plants. Testing

populations of plants transformed with each construct allows the recognition of construct-specific effects and tissue culture-induced effects. Our results open the way to many interesting questions concerning the nature of this resistance and the feasibility of pursuing CP-mediated resistance in this virus system. We are interested in determining whether the resistance is limited to PLRV, or is also effective against other aphid-transmitted or phloem-limited viruses. A common mechanism has been postulated for a genetic resistance to PLRV, PVY, and PVX that was derived from *S. brevidens* (24). Another important question is whether the resistance observed in our control transformants is specific to the T-DNA construct used. To the best of our knowledge, increased virus resistance from a control construct has not been reported, but inclusion of replicates of control construct data has not been emphasized in experiments published to date. Another crucial question that may shed some light on the mechanism of this resistance is whether the lower virus titers are the result of a reduction in infected phloem cells or reduced accumulation of virus in an equal number of infected cells. In an attempt to answer this question we have developed an immunohistochemical technique, utilizing monoclonal anti-PLRV antibodies and a red indicator dye (Vector Red), that allows us to assess the number of infected phloem cells in cross sections of potato stems and leaves in a manner similar to that of Derrick and Barker (8).

Regardless of what the mechanism of this resistance may be, the economic viability of these transformants cannot be assessed until field trials show that this resistance is effective in the field. Factors such as the transformants' susceptibility to net necrosis, yield, and effect on disease epidemiology are currently being addressed in field trials.

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