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Resistance in Transgenic Potato Expressing the Potato Leafroll Virus Coat Protein Gene

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The coat protein gene of potato leafroll virus (PLRV), a member of the luteovirus group, was inserted into potato via a gene transfer mediated by *Agrobacterium tumefaciens*. Introduced as a chimeric gene under the control of the duplicated cauliflower mosaic virus 35S promoter, transcription levels were high but only low levels of coat protein were detected. Coat protein accumulation ranged from undetectable levels to a maximum of 0.01% of total leaf protein. Inoculation of transgenic plants with PLRV resulted in low virus titers that remained low or decreased indicating sustained resistance. High PLRV inoculation levels achieved with 25 viruliferous aphids did not overcome the resistance. This resistance will have practical applications for the control of PLRV and may also help to understand the mechanisms of virus infection.

Additional keywords: genetically engineered resistance, tobacco.

Potato leafroll virus (PLRV), a member of the luteovirus group, causes serious economic losses of potato by reducing both the quality and yield. This virus has a worldwide distribution and is found in most potato-growing areas. It is transmitted by several aphid species in a circulative nonpropagative manner and may be disseminated with infected potato tubers used for seed. These methods of transmission have made control of this disease difficult, costly, and only partially effective (Harrison 1984).

Hamilton (1980) suggested that virus resistance could be obtained by transferring a portion of a viral genome into a plant. Several studies have since indicated that the expression of the coat protein gene of a specific virus in transgenic plants provides resistance toward the virus. Various levels of protection were obtained against tobacco mosaic virus (TMV) (Powell Abel *et al.* 1986), alfalfa mosaic virus (Tumer *et al.* 1987; Loesch-Fries *et al.* 1987; Van Dun *et al.* 1987), potato virus X (PVX) (Hemenway *et al.* 1988), cucumber mosaic virus (Cuozzo *et al.* 1988), tobacco streak virus (Van Dun *et al.* 1988), tobacco rattle virus (Van Dun and Bol 1988), soybean mosaic virus (Stark and Beachy 1989), and potato virus Y (PVY) (Lawson *et al.* 1990). Protection was characterized by a reduced rate of systemic infection, fewer disease symptoms, and lower virus titers. A model has been proposed in which the expression of the TMV coat protein gene in transgenic plant cells prevents TMV particles from uncoating (Register and Beachy 1988). There are, however, some differences in the resistance observed for different viruses. Unlike TMV and alfalfa mosaic virus, the genetically engineered resistance to PVX protected against inoculation with PVX RNA as well as virions. PVY resistance was greatest in those plants that expressed lower levels of coat protein. For plants expressing the coat protein of cucumber mosaic virus, reduced symptom development and virus accumulation occurred independent of the level of inoculum.

PLRV differs from other viruses for which coat protein genes have been used to date to engineer protection in transgenic plants. It is a phloem-limited spherical virus that occurs at low levels in its host and is not transmitted mechanically. This virus, which spreads systemically, has a limited host range restricted mainly to the Solanaceae. Virions of PLRV are composed of a positive sense single-stranded RNA species of 5,883 nucleotides, which includes a single coat protein gene encoding 208 amino acids (Keese *et al.* 1990). The PLRV coat protein open reading frame (ORF) is located near the center of the genome and is thought to be expressed via subgenomic RNA (Mayo *et al.* 1989). The coat protein of luteoviruses has been reported to be responsible for serological properties (Waterhouse and Murant 1981), transmission specificity (Massalski and Harrison 1987; Rochow and Carmichael 1979), cross-protection (Harrison 1958; Webb *et al.* 1952), and mutual exclusion (Jedlinski and Brown 1965).

In an attempt to obtain coat protein-mediated protection, the coat protein gene of PLRV, which was previously identified and sequenced (Kawchuk *et al.* 1989), was inserted into the genomes of both tobacco and potato. These plants were characterized, the potato plants challenged with PLRV using viruliferous aphids, and virus titers monitored using ELISA. The results were compared to those of the transgenic plants expressing the coat protein of other viruses.

MATERIALS AND METHODS

Production of transgenic plants. A cDNA clone of PLRV containing the cistron encoding the coat protein had previously been characterized (Kawchuk *et al.* 1989). From this clone, a DNA fragment (Fig. 1) extending 12 nucleotides 5′ to the AUG start codon and 112 nucleotides beyond

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the 3′ amber codon of the PLRV coat protein gene (designated LCP31) was excised, gel purified, and inserted into the intermediate expression plasmid pCDX1. This vector, which possesses a duplicated cauliflower mosaic virus 35S enhancer-promoter (Kay *et al.* 1987), was inserted into the disarmed octopine-type tumor-inducing (Ti) plasmid pTiB6S3-SE by homologous recombination as described previously (Rogers *et al.* 1986). Recombinant *Agrobacterium tumefaciens* (Smith and Townsend) Conn were selected on media containing 25 μg/ml chloramphenicol, 100 μg/ml spectinomycin, and 50 μg/ml kanamycin.

Leaf disks from *Nicotiana tabacum* L. cv. Xanthi-nc, which were used as a model transformation system, and tuber disks from *Solanum tuberosum* L. cv. Desiree were inoculated with cultures of recombinant *A. tumefaciens* and cultured *in vitro* as described by Horsch *et al.* (1985) and Stiekema *et al.* (1988), respectively. Potato cells carrying integrated copies of the NPTII gene were selected by the growth of calli and subsequent shoot development in the presence of 100 μg/ml of kanamycin. Shoots 3 to 5 mm were excised and rooted in Murashige and Skoog media (Murashige and Skoog 1962) lacking kanamycin. Plants approximately 5 cm in height were transferred to soil prior to analysis.

Analysis of the PLRV coat protein gene in transgenic plants. Leaves frozen with liquid nitrogen were powdered with a mortar and pestle before suspension in a buffer containing 100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), and 0.5% 2-mercaptoethanol. Nucleic acids were extracted twice with equal volumes of phenol and chloroform, then once with chloroform, and were precipitated with sodium acetate and ethanol. The pellet was dissolved, and RNA was purified by precipitation with 2.5 M LiCl. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose (Boehringer Mannheim, Laval, Quebec) column chromatography as described by Aviv and Leder (1972).

Southern hybridization analysis was performed on *Hin*dIII-digested genomic plant DNA that was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) and probed as described by Southern (1975). Isolated plant poly(A)$^+$ RNA for the northern hybridization RNA analysis was run on a methyl mercury denaturing gel, transferred to Nytran membranes, and probed according to the manufacturer's instructions. Randomly primed ^{32}P-labeled DNA probes were prepared against a gel-purified *Alu*I restriction fragment of LP79 containing the coat protein ORF. These probes, which are specific for the PLRV coat protein cistron, were used for both the Southern and the northern blots.

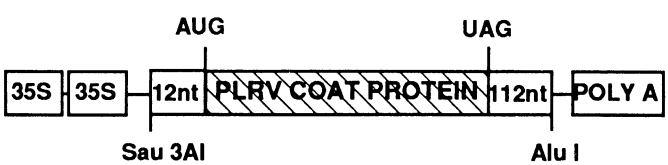

Fig. 1. Diagrammatic representation of the chimeric gene constructed to express the potato leafroll virus (PLRV) coat protein gene. PLRV cDNA fragment LCP31 consists of a 12-nucleotide untranslated leader sequence that precedes the 627-nucleotide coat protein open reading frame and the 112 nucleotides occurring beyond the amber codon of the coat protein gene. Also shown are the duplicated cauliflower mosaic virus 35S promoter and the nopaline synthase polyadenylation signal that provide the control regions for the expression of the PLRV coat protein sequence.

Total phenol-soluble proteins were extracted from plant tissue following the protocol of Van Etten *et al.* (1979). During the final acetone wash, a 1:15 aliquot was spun down, dried with nitrogen, and dissolved in 200 μl of 0.1 M NaOH before incubating 30 min at 60° C. Protein was assayed by the method of Bradford (1976) using the Bio-Rad (Richmond, CA) protein assay. The remaining acetone precipitate was dried with nitrogen and resuspended at a concentration of 15 μg/μl of protein in 10% 2-mercaptoethanol, 4% SDS, 30% glycerol, and 10% bromophenol blue. Samples were placed in boiling water for 5 min, and 150 μg per lane was loaded onto a 12.5% SDS-polyacrylamide gel (Laemmli 1970) with a 4% stacking gel.

Polyacrylamide protein gels were blotted, at 100 V for 2 hr, onto an Immobilon-P membrane (Millipore, Bedford, MA). The western blots were blocked overnight at 4° C with 1% nonfat dried milk in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.1% sodium azide, and 1% bovine serum albumin. PLRV monoclonal ascitic fluid from hybridoma 371A diluted 1:5,000 in the blocking buffer was used to probe the western blots. Blots were washed four times with phosphate-buffered saline (PBS) containing 0.1% Tween 20, incubated with 10^6 cpm/ml of ^{125}I chloramine T-labeled (Hunter and Greenwood 1962) goat anti-mouse antiserum, and again washed four times. Autoradiograms of Southern, northern, and western blots were obtained with overnight exposures.

Challenge of transgenic plants with PLRV. Accumulation of PLRV within the transgenic potato plants was assayed by ELISA. Microtiter wells were coated with (1 μg/ml) IgG from PLRV polyclonal antiserum in 0.05 M carbonate, pH 9.6, followed by blocking with 0.2% nonfat dried milk in PBS with 0.05% Tween 20. Leaf samples were homogenized 1:10 (w/v) in PBS containing 0.2% nonfat dried milk, 0.05% Tween 20, and 2% polyvinylpyrrolidone. One hundred microliter aliquots were loaded per well and incubated overnight at room temperature. Plates were washed and treated with 200 ng/ml of PLRV monoclonal 371A in PBS-Tween 20 for 3 hr at 37° C. Microtiter wells were washed before adding goat anti-mouse antibodies conjugated to alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, MD) for 1 hr at 37° C. Plates were washed again, and 100 μl of *p*-nitrophenylphosphate at 0.5 mg/ml in 10% diethanolamine, pH 9.8, was added to each well. Absorbance at 405 nm (A_{405}) was recorded after 1 hr and overnight incubations at room temperature with a Titertek Multiscan 8 channel plate reader (Flow Laboratories, McLean, VA). The plate reader was blanked on a row treated as described above but without samples. Controls were placed on each plate and included PLRV-infected tissue and healthy uninoculated tissue from untransformed plants.

Transgenic plants D2, D7, D8, and D12 of potato cultivar Desiree that were to be inoculated with PLRV were vegetatively propagated by stem cuttings, placed in 6-inch diameter pots, and grown in a greenhouse with a 16-hr

day and 8-hr night cycle. Control plants consisted of vegetatively propagated cultivar Desiree not transformed by *Agrobacterium*. Plants were inoculated with PLRV using the aphid vector *Myzus persicae* Sulz., which had fed previously on *Physalis pubescens* L. infected with PLRV. Vegetatively propagated D2, D7, D8, D12, and untransformed cultivar Desiree were inoculated with PLRV by placing five aphids on various leaves of plants approximately 10 cm in height for 5 days. Plants were inspected during the inoculation period to ensure that the aphids were feeding.

Initially, 18 virus-free vegetative cuttings from each of D2, D7, D8, and a control were inoculated with PLRV. Leaf samples of two or three individual leaves from different locations from each plant were collected and tested by ELISA at 10-day intervals over a 50-day period. The experiment was then repeated using 18 vegetative cuttings from each of D2, D8, D12, and a control. Leaf samples were collected biweekly over a 72-day period following the challenge inoculations.

To establish the effect of the inoculum level, each of eight vegetatively propagated cuttings from transgenic cultivar Desiree D12 and control Desiree were inoculated using either 1, 5, or 25 viruliferous *M. persicae*. Leaf samples were collected and tested periodically over a 78-day period.

RESULTS

Transformation efficiency. From 91 cultivar Desiree tuber disks inoculated with *A. tumefaciens*, 12 shoots were isolated from independent calli. Many more shoots were obtained from the tobacco transformation. All isolated potato shoots and three tobacco shoots that rooted in 100 μg/ml of kanamycin were further characterized before being selected for virus challenge. With the exception of D2, transgenic potato and tobacco plants showed no observable phenotypic response to the insertion of the PLRV coat protein gene or the presence of the transcript. Transgenic D2 showed a minor alteration in leaf morphology with leaves being narrower than those of control Desiree. Each transgenic potato plant produced viable tubers, and the tobacco plants gave fertile R1 progeny by self-fertilization. Vegetatively propagated potato plants provided the means of monitoring a large sample size of phenotypically and genetically identical cuttings.

Southern analysis. Digestion of transgenic plant genomic DNA with *Hin*dIII included fragments of approximately 1,100 nucleotides that contained the 751 nucleotides (LCP31) of the inserted PLRV DNA sequence shown in Figure 1. Southern analysis of cultivar Desiree D2 and D12 and parental tobacco T5 indicated different signal strengths of the LCP31 insert in the plants (Fig. 2). Similar

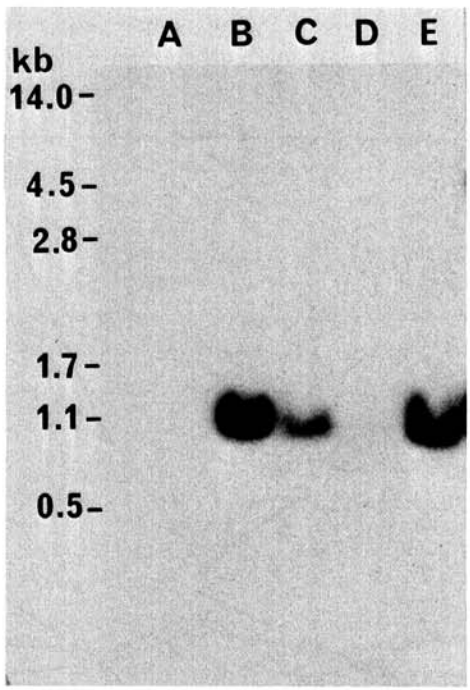

Fig. 2. Southern blot analysis of *Hin*dIII-digested DNA from plants transformed with the potato leafroll virus (PLRV) coat protein gene. The autoradiograph shows a *Hin*dIII fragment of approximately 1,100 nucleotides in transgenic potato D2 and D12 and transgenic tobacco T5 (lanes B, C, and E, respectively), but not in untransformed potato and tobacco (lanes A and D, respectively). The blot was probed with randomly primed DNA prepared from a gel-purified *Alu*I fragment containing the PLRV coat protein gene.

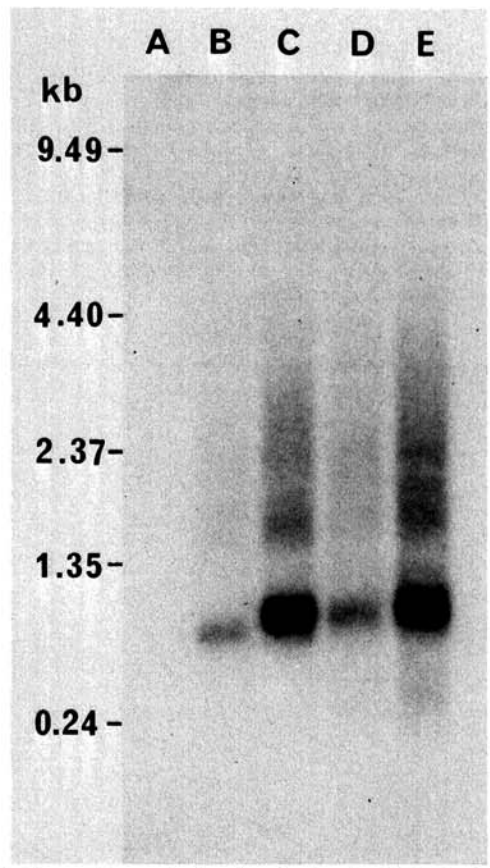

Fig. 3. Northern blot analysis of polyadenylated RNA from plants transformed with the potato leafroll virus (PLRV) coat protein gene. A polyadenylated transcript with an approximate length of 1,000 nucleotides was observed in the autoradiograph of both transformed potato (D2), lanes B and C (0.3 and 0.9 μg total polyadenylated RNA, respectively), and transformed tobacco (T5), lanes D and E (0.3 and 0.9 μg total polyadenylated RNA, respectively). No homologous transcript was observed in 0.9 μg of total polyadenylated RNA of untransformed potato (lane A). Blots were probed with randomly primed DNA prepared to a gel-purified *Alu*I fragment containing the PLRV coat protein gene.

analysis (data not shown) was used to confirm that all plants examined contained the appropriate DNA insert.

Transcript analysis. Northern blots (Fig. 3) of potato D2 and tobacco T5 detected high levels of the poly(A)$^+$ PLRV coat protein transcript. Comparison of the signal intensity of known quantities of PLRV RNA to that of the detected transcript suggested that the transgenic plants (Fig. 3, lanes B and C) contained approximately 10 ng of PLRV transcript for each microgram of total polyadenylated RNA. Similar analysis of control Desiree showed that it had no detectable coat protein transcript (Fig. 3, lane A). The LCP31 transcripts were approximately 1,000 nucleotides long, which is the expected length if the transcripts have a poly(A)$^+$ tail of approximately 250 nucleotides. The transcripts appeared to be relatively stable as little degradation product was observed.

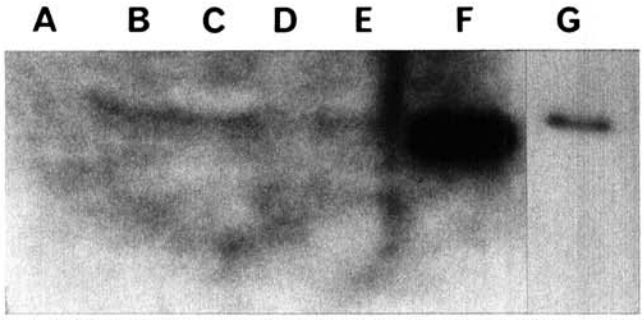

Fig. 4. Western blot analysis of potato leafroll virus (PLRV) coat protein expression in transgenic potato plants. Total phenol-soluble proteins were extracted from the leaves of transgenic potato D2, D7, D8, and D12 (lanes B, C, D, and E, respectively) and untransformed potato (lane A) as a control. Total protein was quantified, and 150 μg was loaded per lane, separated, blotted, and probed using monoclonal 371A and ^{125}I-labeled goat anti-mouse antiserum. The blot also shows 30 μg of total phenol-soluble protein extracted from an untransformed plant infected with PLRV (lane F) and 20 ng of purified virus (lane G), which represents approximately 14 ng of coat protein.

Detection of coat protein. Western analysis of transgenic potato plants indicated low levels of protein expression (Fig. 4). The extraction of phenol-soluble proteins facilitated higher loadings and the detection of lower levels of protein as compared to extracts prepared according to the method of Powell Abel *et al.* (1986). Among the plants of cultivar Desiree tested, the amount of PLRV coat protein varied from undetectable amounts to approximately 0.01% of the total protein, with D2 expressing the highest levels. The protein produced by the transgenic plants comigrated with the PLRV coat protein subunit from purified virus.

ELISA failed to detect any expressed coat protein subunit in the transgenic plants. This permitted the ELISA protocol as described above to be used for screening virus levels in plants inoculated with PLRV.

PLRV resistance. Tissue that was not inoculated with PLRV gave readings between 0 and 0.02 absorbance at 405 nm. Shortly after inoculation using five viruliferous aphids, PLRV levels within both control and transgenic plants were monitored periodically by ELISA (Fig. 5A). During the initial stages of infection, virus levels in the control and transgenic plants were similar, but even at this early stage the average PLRV titers were lower in the transgenic plants. As the infection proceeded, the PLRV titers in the control plants increased rapidly after 30 days, whereas in the transgenic plants the titers remained at low levels and appear to have decreased 40 to 45 days after inoculation to lower or undetectable levels. The overall response of each transgenic cultivar Desiree line was similar.

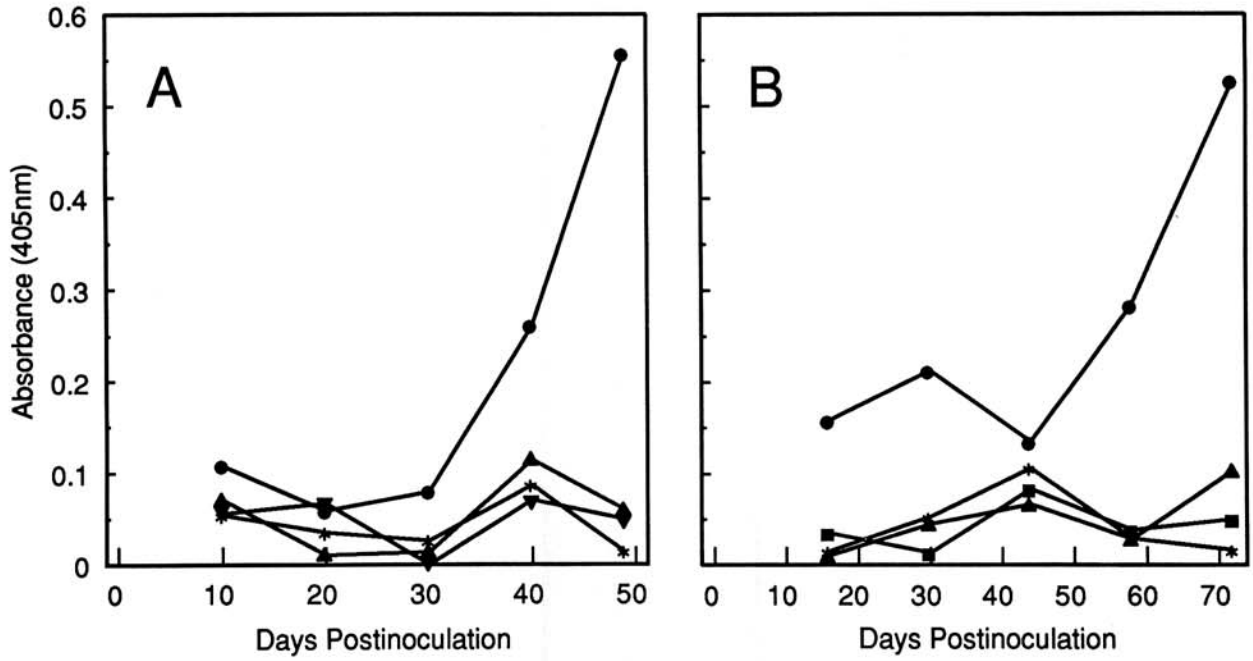

Fig. 5. Average relative virus titers in plants inoculated with potato leafroll virus using five viruliferous aphids. Average ELISA (A_{405}) values were determined for 18 plants each of transgenic lines D2 (▲), D7 (▼), D8 (*), and D12 (■), with untransformed potato (●) as a control. Uninoculated plants gave readings between 0 and 0.02 at A_{405}. Results in **A** were obtained under the same conditions as those in **B**, but represent two independent experiments.

Both transgenic and control potato plants failed to develop any primary infection symptoms, which are usually absent in greenhouse-grown potato plants.

Repetition of the experiment (Fig. 5B) indicated that relatively low levels of virus were detected in the transgenic plants, and a decrease of virus titers may have occurred within these plants between 40 and 45 days after inoculation. A slight increase of virus titer in transgenic D2 was noted after almost 3 months. However, the low virus titers in the transgenic plants were very similar in both tests and were estimated as less than 10% of the control plants (as determined below).

An increase in inoculum of PLRV did not overcome the observed resistance achieved in the transgenic plants. Inoculation with 5 or 25 viruliferous aphids (Fig. 6, B and C) quickly resulted in higher average virus titers in the control plants as compared to control plants inoculated by one aphid (Fig 6A). However, transgenic D12 possessed similar low average virus titers regardless of being inoculated by 1, 5, or 25 viruliferous aphids. Dilution series response curves (data not shown) for the control and transgenic Desiree (D12) inoculated by 25 viruliferous aphids indicated that the level of virus in the transgenic plants was less than 10% of that in the control plants.

Using five viruliferous aphids for inoculation, 13 and 14 of the 18 control plants became infected in the first and second tests, respectively. Similar results were observed with the control plants used to investigate inoculum levels. No escapes occurred when 25 aphids were used to inoculate each plant, but at five aphids per plant two escapes occurred and little or no virus was detectable in those plants inoculated by one aphid. Virus titers in infected control plants increased to high levels and could be easily distinguished from escapes, but this was not true for transgenic plants. They were not defined as infected or uninfected since the virus titers were low (estimated $<10\%$ of controls) and varied slightly between tests. However, based on the number of escapes observed with control plants, a similar number may be expected when using five aphids for inoculation of transgenic plants.

At first virus was detected in some individual plants and not others regardless of whether they were transformed or control. The number of control plants that tested for PLRV increased with time, whereas the number of transgenic plants that tested positive remained the same or decreased. Titers in the transgenic plants remained low with the highest titers being well below those in individual control plants. As the plants matured, the virus levels in control plants increased to high levels, but remained the same or decreased to low or undetectable levels in the transgenic plants.

DISCUSSION

The PLRV coat protein gene was successfully inserted into potato cultivar Desiree, and high levels of sustained resistance to PLRV infection were obtained. Investigations of other viruses (Powell Abel *et al.* 1986; Tumer *et al.* 1987; Loesch-Fries *et al.* 1987; Van Dun *et al.* 1987; Hemenway *et al.* 1988; Cuozzo *et al.* 1988; Van Dun *et al.* 1988; Van Dun and Bol 1988; Stark and Beachy 1989; Lawson *et al.* 1990) have shown resistance to infection in plants expressing the specific coat protein gene. However, several features observed in this study may be unique to PLRV and possibly other luteoviruses.

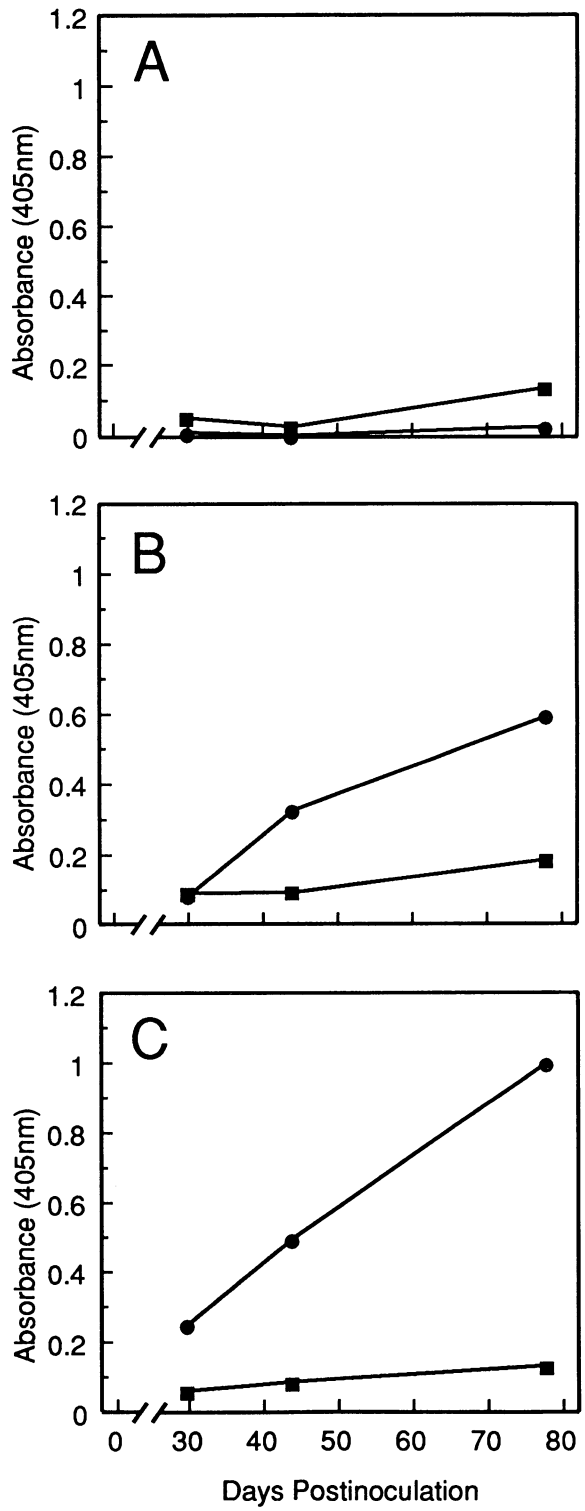

Fig. 6. Relative virus accumulation in plants after inoculation with potato leafroll virus using 1 (**A**), 5 (**B**), or 25 (**C**) viruliferous *Myzus persicae*. Each point represents the average ELISA (A_{405}) values in eight plants. Results at each inoculum level are reported for transgenic potato line D12 (■) and untransformed potato control Desiree (●).

Levels of PLRV coat protein transcripts were relatively high in the transgenic plants as predicted using the duplicated cauliflower mosaic virus 35S enhancer for expression. However, coat protein represented less than 0.01% of the total leaf protein. This may be a result of low translational efficiency of coat protein RNA. According to certain consensus sequences identified for translational efficiency, the sequence surrounding the PLRV coat protein AUG start codon may not promote high levels of translation (Lutcke *et al.* 1987); however, the native virus has the same sequence. Another possibility is that the untranslated region 5′ to the PLRV coat protein is necessary for efficient translation of the coat protein. The construct LCP31 (Fig. 1) includes only 12 nucleotides from this 197-nucleotide untranslated region adjacent to the coat protein ORF. Alternatively, the low level of coat protein may be a result of the protein subunit being unstable or stable only within specific tissues such as phloem cells to which the native virus is confined.

If the coat protein is imparting resistance, then it must be able to do so at extremely low levels or possibly be concentrated in specific cells such as those of the phloem. Transgenic plants expressing low levels of PVY coat protein were able to provide specific resistance toward PVY (Lawson *et al.* 1990). Alternatively, protection may be achieved by the transcript produced in the transgenic plant that may interfere at some stage of viral replication.

The method of inoculation used to infect plants in these experiments is the same mechanism that is responsible for movement in the field. Most other studies of viral coat protein expression in transgenic plants have challenged the plants using mechanical inoculations. While this allows for more precise quantitation of the inoculum, it may not reflect the level of protection observed when the virus is transmitted by its natural vectors. An aphid-transmitted virus is deposited at a low level on a leaf basis, but at the level of the cell the level of inoculum may be at a much higher concentration than that obtained by mechanical transmission. Thus, virus transmission to transgenic plants by the natural vector would provide the best indication of the effectiveness of the genetically engineered resistance in the field.

Since luteovirus levels have been reported to vary among different leaves on the same plant (Pereira and Lister 1989), these effects were minimized by sampling two leaves from every plant at each sampling date. Although a decline of virus was observed in the clones from all four transgenic lines during both experiments and occurred 40 to 45 days following inoculation, further analysis is required to determine its significance. This pattern of decline in virus concentration once replication has begun has not been reported in plants expressing other coat protein genes. With other viruses, once the transgenic plant became infected the protection was not as effective.

An increase in the level of PLRV inoculum did not overcome the observed resistance. Young potato plants, similar to those inoculated, represent the tissue that is most susceptible to PLRV infection (Knutson and Bishop 1964). This together with the high inoculum levels obtained with 25 viruliferous aphids exceeds a heavy field infestation. The ability of the plants to achieve resistance under these extreme conditions indicates that they offer a practical means of virus control. Although virus was not excluded from every transgenic plant, it declined to very low or undetectable levels. Thus the quality and yield of tubers should be improved and aphid transmission reduced or effectively eliminated. Further modifications of the inserted gene could be made to increase or decrease the level of transcript or coat protein and maybe increase the level of resistance obtained. In view of the sequence similarity of the three luteoviruses sequenced to date, PLRV (Mayo *et al.* 1989; Van der Wilk *et al.* 1989; Keese *et al.* 1990), beet western yellows virus (Veidt *et al.* 1988), and barley yellow dwarf virus-PAV (Miller *et al.* 1988), it is likely that this strategy may be used to obtain resistance with other luteoviruses.

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