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Genetically Engineered Resistance to Potato Virus S in Potato Cultivar Russet Burbank

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The coat protein gene from potato virus S (PVS) was introduced into potato cultivar Russet Burbank by leaf disk transformation using *Agrobacterium tumefaciens*. Transgenic plants expressing the viral coat protein were resistant to subsequent infection following mechanical inoculation with the Andean or ME strain of PVS as indicated by a lack of accumulation of virus in the upper leaves. In agreement with previous reports for plants expressing potato virus X coat protein, plants expressing PVS coat protein were also protected from inoculation with PVS RNA, and in addition they also showed a measure of resistance to inoculation with a related carlavirus, potato virus M. The coat protein-mediated protection afforded by these transgenic plants was sufficient to prevent the accumulation of virus in the tissues of nontransformed Russet Burbank shoots that had been grafted onto transgenic plants inoculated with PVS. In reciprocal grafts, shoots from transgenic plants contained significantly lower virus concentrations following grafting onto plants systemically infected with PVS.

Additional keywords: transgenic potato, virus resistance.

Potato is one of the most agronomically important crops worldwide. However, because of the tetraploid nature of its genome, classical approaches to potato breeding and selection for improved properties are especially difficult and laborious when compared with other diploid species. Even in light of these drawbacks, significant progress has been achieved over many years, and specific cultivars have been developed with desirable characteristics. Russet Burbank is one of the oldest and most popular of the potato cultivars grown in the continental United States and Canada. While Russet Burbank has excellent processing characteristics and high yield, it lacks resistance to many plant pathogens including bacteria, nematodes, and many plant viruses. Because the propagation of potato is almost exclusively vegetative, many of these diseases can be perpetuated over time with disastrous results.

Potato virus S (PVS) is a member of the carlavirus group and is aphid-transmitted in a nonpersistent manner to members of the Solanaceae and Chenopodiaceae. The viral RNA is encapsidated in a coat protein with an M_r of 33,000 into slightly flexuous 650 × 12 nm filamentous particles (Koenig 1982). The viral genome consists of one single-stranded, positive-sense RNA molecule with an estimated M_r of 2.4×10^6 that contains a 3′-terminal polyadenylated region. *In vitro* translation of the Andean (An) strain of PVS (PVS-An) RNA in rabbit reticulocyte lysates has been reported to yield three high molecular weight polypeptides (M_r = 140K, 123K, and 104K) in addition to the 33K capsid protein (Monis and De Zoeten 1990). The viral genome of PVS contains six open reading frames (ORFs) that encode polypeptides with M_r of 10,734, 32,515, 7,222, 11,802, 25,092, and more than 41,052 (MacKenzie *et al.* 1989). This latter ORF encodes amino acid sequences similar to those of putative viral replicase genes, and the polypeptide with an M_r of 32,515 has been shown to be the virus coat protein. The biological functions of the 7K, 12K, 25K, and 3′-terminal 11K putative nonstructural proteins remain to be determined. While the molecular mechanisms of PVS disassembly and/or assembly in infected cells are unknown, it is believed that the viral coat protein is translated from an encapsidated subgenomic RNA species of approximately 1.3 kilobases (Foster and Mills 1990). The presence of encapsidated subgenomic RNAs encoding the viral coat protein has also been reported for at least two members of the potexvirus group, white clover mosaic virus (Forster *et al.* 1987) and narcissus mosaic virus (Short and Davies 1983).

Because of its near symptomless morphology in potato, PVS has been a difficult virus to control and can occur in seed lots at levels of 70% or more (MacKinnon and Bagnall 1972). In combination with potato virus X (PVX), it can cause significant reduction in tuber yields (Wright 1977). Elite seed stocks in Canada are produced from virus-free material by heat therapy and meristem tip culture (Wright 1987), but such stocks become rapidly reinfected with PVS once grown in the field (Hahm *et al.* 1981). Contributing to this is the fact that many potato viruses, including PVS, overwinter in tubers missed in the previous harvest, and volunteer plants emerging from such tubers are a primary source of inoculum and play a significant role in virus epidemiology.

Since the first report of the expression of bacterial genes in plants (Fraley *et al.* 1983), the process of genetic transformation and regeneration, as a mechanism for the expres-

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sion of novel genes, has become routine for a number of plant species (Gasser and Fraley 1989). However, the application of these techniques for the genetic engineering of desirable resistance traits into agronomically important potato cultivars has only become feasible in recent years with the advent of efficient tissue culture techniques for the regeneration of transformed potato tissue (Stiekema *et al.* 1988; De Block 1988).

The first successful demonstration of genetically engineered resistance to a plant virus was reported by Powell-Abel *et al.* (1986), who found that transgenic tobacco plants expressing the coat protein gene from tobacco mosaic virus (TMV) showed a significant delay in symptom development following inoculation with TMV. This form of protection mediated by the coat protein has now been demonstrated for a number of different viruses from at least seven different virus groups and has been the subject of a recent review (Beachy *et al.* 1990). While the coat protein-mediated protection observed in transgenic plants infected with these viruses has characteristics similar to the phenomenon of cross-protection (Hamilton 1980; Fulton 1986), it remains to be determined to what extent the underlying biological mechanisms are related. Engineered resistance is generally specific to the homologous virus or related viruses sharing a high degree of coat protein homology (Van Dun and Bol 1988; Nejidat and Beachy 1990) and, at least in the case of TMV, requires the accumulation of coat protein rather than RNA sequences coding for coat protein (Powell *et al.* 1990).

We have previously reported that the expression of PVS coat protein in transgenic *Nicotiana debneyi* results in protection against PVS infection (MacKenzie and Tremaine 1990). In this report we describe the transformation and regeneration of Russet Burbank potato plants expressing PVS coat protein that are resistant to inoculation with PVS or purified PVS RNA and show a measure of resistance to infection by potato virus M (PVM).

MATERIALS AND METHODS

Virus purification and preparation of viral RNA. The Peruvian (Andean) strain of PVS (Hinostroza-Orihuela 1973) was purified from systemically infected *Chenopodium quinoa* as described previously (MacKenzie *et al.* 1989). A local isolate of PVM was purified from infected potato using a similar protocol. Viral RNA was prepared from particles treated with alkaline sodium dodecyl sulfate (SDS) by multiple phenol-chloroform extractions and ethanol precipitation.

Construction of pVS153 plant expression vector and potato transformation. The preparation and characterization of a complementary DNA (cDNA) clone (pVS57), which contains the coding sequence of the entire PVS coat protein gene inserted at the *Sma*I site of pUC13, as an in-frame fusion with the *lacZ* α-peptide, have been described previously (MacKenzie *et al.* 1989). Digestion of pVS57 with *Hin*dIII yielded a fragment of 1,216 base pairs whose 5′ terminus was located 16 nucleotides upstream from the initiating ATG codon for the 33K viral coat protein gene and whose 3′ terminus was located 68 base pairs upstream of the poly(A) tract at the 3′ end of the PVS RNA sequence. This fragment was subcloned into the *Hin*dIII-digested pBluescript KS+ vector (Stratagene, La Jolla, CA), and an appropriately oriented *Eco*RI-*Xho*I fragment from one of these resulting clones was subsequently transferred to an intermediate, cointegrate tumor-inducing (Ti) plasmid vector, pCDX1, which had been digested with *Eco*RI and *Xho*I. Vector pCDX1 (Kay *et al.* 1987) is a derivative of pMON178 and contains a duplicated cauliflower mosaic virus 35S promoter upstream from a multiple cloning site and the nopaline synthase polyadenylation signal. This construct was then introduced into the resident disarmed octopine-type plasmid, pTiB6S3SE, carried by strain GV3111SE of *Agrobacterium tumefaciens* by homologous recombination using a triparental mating procedure, and the resultant clones were isolated by screening for resistance to spectinomycin, kanamycin, and chloramphenicol (Rogers *et al.* 1986).

Leaf explants from Russet Burbank potato grown *in vitro* were wounded and cocultivated with *A. tumefaciens* GV3111SE carrying the pTiB6S3SE-pVS153 Ti plasmid construct, and transformed shoots were regenerated under kanamycin selection following the procedure of De Block 1988. Shoots (0.5–1.0 cm in height) were then rooted in the presence of 2 mg/L of indoleacetic acid and after 2–3 wk transferred to soil.

Western blot analysis of coat protein expression. The ability of transgenic Russet Burbank potato to produce PVS coat protein was assessed by western immunoblotting using a polyclonal rabbit antiserum specific to the viral capsid protein. Samples of leaf tissue (100 mg) were homogenized with 100 μl of SDS-PAGE sample buffer (4% SDS, 125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 0.04% bromophenol blue, 20% glycerol) and incubated at 95° C for 5 min. Following centrifugation at 13,000 rpm $\times$ 5 min in an Eppendorf microcentrifuge, 15-μl aliquots were loaded onto a 12% polyacrylamide gel, and after electrophoresis using the buffer system of Laemmli (1970), separated proteins were blotted onto an Immobilon membrane (Millipore, Bedford, Ma) in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, at 100 V (0.25 A) for 60 min at 4° C.

The transfer blots were treated with blocking buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, 0.1% NaN_3) for 60 min and incubated with purified rabbit anti-PVS IgG (10 μg/ml in blocking buffer) for 60–90 min. Following rinsing with phosphate-buffered saline plus 0.1% Tween 20, blots were incubated with ^{125}I-labeled protein A (1.5×10^6 cpm/μg; 1.0×10^6 cpm/ml in blocking buffer) for 60–90 min followed by exhaustive rinsing with phosphate-buffered saline-Tween and overnight autoradiography (−70° C).

Enzyme-linked immunosorbent assay (ELISA). Samples of leaf tissue (100 mg) from kanamycin-resistant, regenerated Russet Burbank shoots in tissue culture were initially screened for expression of the PVS coat protein using a double-antibody sandwich (DAS) ELISA. Tissue samples were homogenized with 200 μl of blocking buffer containing 1 mM phenylmethylsulfonyl fluoride as a protease inhibitor using a stainless steel pestle fitted to an Eppendorf microcentrifuge tube (1.5-ml-diameter). Microtiter plate wells were coated with 100 μl of purified rabbit anti-PVS

IgG (20 μg/ml in 50 mM sodium carbonate, pH 9.6) and incubated overnight at 4° C. Wells were then rinsed with water, treated with blocking buffer for 60 min, and incubated with 100-μl volumes of tissue homogenate in blocking buffer overnight at 4° C. Following rinsing, wells were incubated sequentially with 100 μl of alkaline phosphatase-conjugated rabbit anti-PVS IgG (200 ng/ml in blocking buffer; 90 min) and *p*-nitrophenyl phosphate substrate (0.5 mg/ml in 10% diethanolamine, pH 9.8; 2 hr at 24° C). The absorbance of each well was measured at 405 nm using a Titertek Multiskan MC multiwell plate reader (Flow Laboratories, McLean, VA) interfaced with an IBM PC/AT microcomputer. Transgenic plantlets that had been transferred to soil and which tested positive by ELISA were subsequently screened by western immunoblotting using polyclonal rabbit anti-PVS IgG and ^{125}I-labeled protein A.

A similar procedure was used for the ELISA detection of virus infection in inoculated plants. Duplicate composite samples, consisting of 6-mm-diameter leaf disks obtained from at least six plants (approximately 108 mg tissue total), were processed through a mechanical leaf press irrigated with 0.5 ml of blocking buffer. Antibody-coated wells were incubated with 100-μl volumes of serially diluted tissue homogenate in blocking buffer overnight at 4° C. Concentrations of PVS in each sample were computed relative to a standard response curve constructed using samples of purified virus (5,000 to 2.45 ng/ml) diluted in blocking buffer.

The concentration of PVM in infected tissue samples was similarly determined by DAS-ELISA, except that microtiter plate wells were initially coated with rabbit anti-PVM IgG (10 μg/ml), and bound virus was detected using a specific monoclonal antibody and alkaline phosphatase-conjugated goat anti-mouse IgG.

Virus inoculation of potato plants. Transgenic and non-transformed potato plants were propagated in tissue culture and transferred to soil. Plants at the four- to six-leaf stage were dusted with Carborundum and inoculated with various dilutions (0.5, 1.0, 2.0, and 5.0 μg/ml in 10 mM sodium phosphate buffer, pH 7.2) of a preparation of PVS-An. Duplicate composite samples, consisting of 6-mm-diameter leaf disks obtained from at least six plants (approximately 108 mg tissue total), were collected at various times after inoculation and assayed for the presence of the PVS coat protein antigen as described previously. In separate experiments, plants were also inoculated with purified viral RNA (2.0 and 5.0 μg/ml) or with a purified preparation of PVM (5.0 μg/ml).

RESULTS

Regeneration and transformation. The PVS coat protein cistron contained in plasmid pVS57, isolated by screening a PVS genomic cDNA library by colony hybridization with a monoclonal antibody specific for the viral capsid protein (MacKenzie *et al.* 1989), was introduced into the intermediate, cointegrate Ti plasmid vector pCDX1 (Kay *et al.* 1987) (Fig. 1). Following integration of this construct into the pTiB6S3SE helper Ti plasmid, carried by *A. tumefaciens* GV3111SE, leaf explants from axenically grown Russet Burbank potato were transformed and regenerated using the method of De Block (1988). The regeneration of transformed Russet Burbank tissue using this protocol was an efficient process and resulted in rooted transgenic shoots in about 8–12 wk. Virtually all of the initial leaf explants gave rise to one or more vigorously growing calli, and more than 90% of these ultimately produced shoots. Of the 85 rooted transgenic shoots generated, approximately half were tested for the expression of PVS coat protein by ELISA, and 70% of these produced detectable levels of viral coat protein. Ten of these coat protein positive (CP^+) transgenic lines, which appeared morphologically normal, were maintained in tissue culture and expanded further.

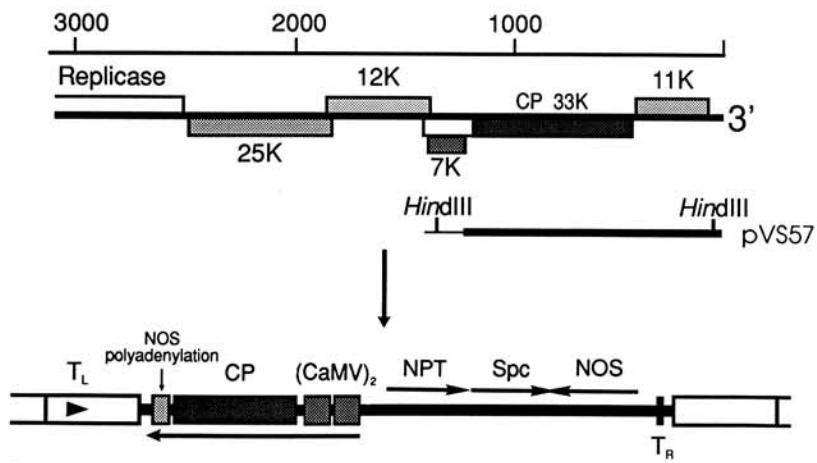

Fig. 1. Genome organization of potato virus S (PVS) RNA and a schematic representation of the arrangement of the coat protein (CP) cistron following triparental mating and homologous recombination into the disarmed pTiB6S3SE plasmid carried by *Agrobacterium tumefaciens* GV3111SE. A *Hin*dIII fragment derived from pVS57 was cloned into the pBluescript KS+ vector and subsequently excised by double digestion with *Xho*I and *Eco*RI for insertion into pCDX1, which had been digested with *Xho*I and *Eco*RI. The left and right T-DNA border sequences are represented by T_L and T_R, respectively, and genes for nopaline synthase (NOS), spectinomycin resistance (Spc), and neomycin phosphotransferase (NPT) are indicated. The arrow below indicates the direction of transcription of the PVS coat protein gene under the control of the duplicated cauliflower mosaic virus (CaMV) 35S promoter.

Expression of PVS coat protein. Western immunoblot analysis was used to confirm the *in vivo* expression of PVS coat protein in regenerated transgenic potato plants. Protein extracts from four different transgenic lines, nontransformed Russet Burbank potato, and different concentrations of purified PVS were separated by SDS-PAGE, and blots were probed with rabbit anti-PVS IgG and ^{125}I-labeled protein A (Fig. 2). Based on comparisons with PVS standards (Fig. 2, lanes 1, 2, and 3), the level of coat protein expression in three of these lines, RB18, RB41, and RB58 (Fig. 2, lanes 5, 6, and 8, respectively), was estimated to be ≤0.1–0.2% of the total SDS-soluble protein present in tissue extracts, while RB52 plants (Fig. 2, lane 7) produced significantly less coat protein.

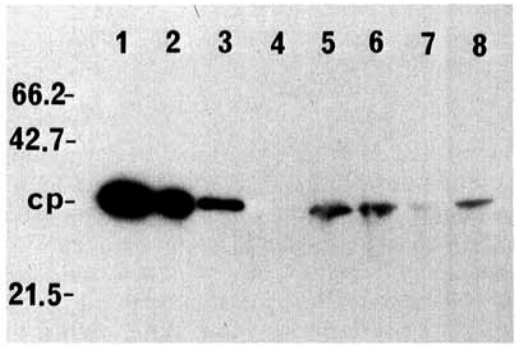

Fig. 2. Western blot analysis of potato virus S (PVS) coat protein expression in transgenic Russet Burbank potato plants. Immunoblots of coat protein standards (100, 50, and 20 ng) from a purified preparation of PVS (lanes 1, 2, and 3) and total sodium dodecyl sulfate-soluble protein (8–10 μg) extracted from leaf tissue of transgenic lines RB18 (lane 5), RB41 (lane 6), RB52 (lane 7), and RB58 (lane 8) or a nontransformed potato plant (lane 4) were incubated with rabbit anti-PVS IgG followed by incubation with ^{125}I-labeled protein A. The migration of PVS coat protein (CP) and M_r standards ($\times 10^{-3}$) are indicated.

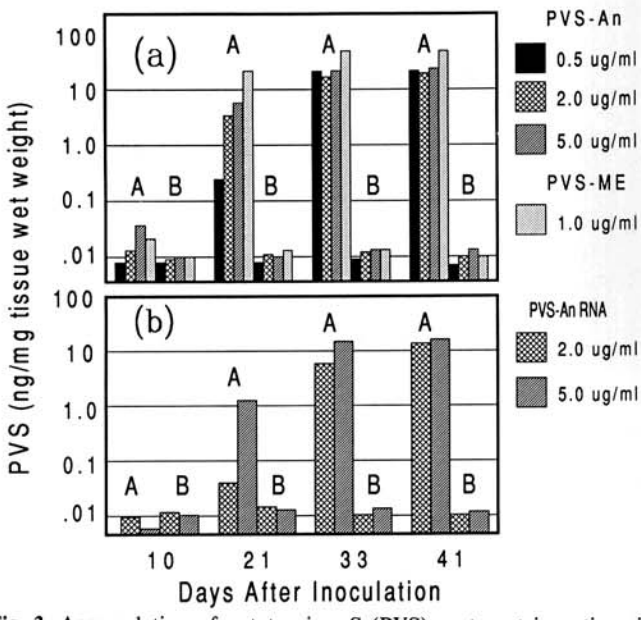

Fig. 3. Accumulation of potato virus S (PVS) coat protein antigen in the upper leaves of nontransformed (A) and RB58 transgenic Russet Burbank potato (B) plants after inoculation with either intact PVS particles (panel a) or PVS RNA (panel b) at various days after inoculation. Plants were mechanically inoculated with preparations of the Andean (An) strain of PVS (PVS-An; 0.5, 2.0, and 5.0 μg/ml), the ME strain of PVS (PVS-ME; 1.0 μg/ml), or PVS-An RNA (2.0 and 5.0 μg/ml). Viral coat protein concentrations were determined by double-antibody sandwich ELISA and are expressed as the $\log_{10}$ of nanograms of virus coat protein per milligram wet weight of tissue.

Susceptibility of transgenic Russet Burbank potato to infection with PVS or viral RNA. Transgenic potato plants expressing the PVS coat protein were challenged by inoculation with purified PVS or PVS RNA. Virus replication and spread were monitored in these plants by sampling disks from the upper uninoculated leaves of each plant at various times after inoculation and quantitating viral antigen levels by DAS-ELISA. By 2–3 wk following inoculation of nontransformed Russet Burbank potato with a purified preparation of PVS-An, measurable quantities of virus could be readily detected in all of the inoculated plants, and after 5 wk the virus titer had reached approximately 20–25 ng/mg wet weight of tissue (Fig. 3, panel a). Plants inoculated with the ME strain of PVS showed a more rapid accumulation of virus and also displayed an approximately twofold higher maximum level of virus when compared with the An strain. Nontransformed plants that had been inoculated with PVS RNA, while showing a slower rate of virus accumulation in systemic leaves when compared with plants inoculated with intact virus particles, did show comparable virus titers by 6 wk after inoculation (Fig. 3, panel b).

In contrast, RB58 transgenic plants failed to accumulate any significant (greater than background) quantities of viral antigen over the course of the experiment (Fig. 3, panel a), even at the highest inoculum level tested (5 μg/ml). In agreement with our previous results with transgenic *N. debneyi* expressing the PVS coat protein, these PVS CP$^+$ potato plants were also resistant to infection with PVS RNA (Fig. 3, panel b). The PVS resistance of four other transgenic potato lines (RB18, RB41, RB52, and RB81), which expressed comparable levels of PVS coat protein,

Table 1. Concentration of potato virus S (PVS) coat protein antigen in transgenic and nontransformed plants of potato cultivar Russet Burbank following inoculation with PVS or PVS RNA

Plant line	Inoculum level (μg/ml)	Days after inoculation	PVS concentration (ng/mg tissue)
RB18	1.0	41	≤0.008[a]
	10.0	41	≤0.004
RB41	1.0	41	≤0.003
	10.0	41	≤0.007
RB52	1.0	41	≤0.004
	10.0	41	≤0.002
RB58	5.0	60	≤0.002
	5.0 (RNA)	60	≤0.003
RB81	10.0	47	≤0.009
	PVS-ME sap[b]	47	≤0.003
Nontransformed	1.0	47	21.49
	10.0	47	21.98
	5.0	60	53.82
	5.0 (RNA)	60	24.35
	PVS-ME sap	47	115.3

[a] Concentrations of PVS coat protein antigen in the range of ≤0.002–0.030 ng/mg of tissue represent the lower limit of detection by double-antibody sandwich ELISA and are equal to the background values obtained from uninoculated transgenic plants.

[b] Plants were inoculated with an extract obtained from potato infected with the ME strain of PVS (PVS-ME) prepared by homogenizing 1 g of leaf tissue with 10 ml of 10 mM sodium phosphate buffer, pH 7.4.

paralleled the results obtained with RB58 plants (Table 1).

The extent of protection offered by transgenic plants expressing the PVS coat protein was further evidenced by the lack of accumulation of PVS in the upper leaves of nontransformed Russet Burbank shoots that had been grafted onto RB41 plants inoculated with purified PVS-An (Table 2).

In separate experiments, transgenic RB41 or RB58 shoots that had been grafted onto nontransformed potato plants systemically infected with PVS also failed to accumulate any significant levels of PVS by 21 days after grafting. Under the same conditions, the concentration of PVS coat protein antigen in nontransformed, initially virus-free shoots similarly grafted onto PVS-infected parent plants was equivalent to that observed in systemically infected Russet Burbank by 21 days after grafting (Table 2). In a similar manner, the ability of PVM to effectively cross-protect against PVS infection was also shown by the lack of accumulation of PVS in the upper leaves of PVM-infected shoots that had been grafted onto PVS-infected parent plants (Table 2). The accumulation of PVS (42 days after grafting) in stem or leaf tissues from various parts of these grafted plants is shown in Figure 4. Tissue samples removed from either the lower or upper stem segments or the apical leaves of transgenic (RB41 and RB58) or PVM-infected shoots grafted onto PVS-infected parent plants showed a marked reduction in the level of PVS antigen when compared with similar samples from plants containing nontransformed grafts. For example, stem segment samples removed from transgenic RB41 and RB58 or PVM-infected grafts just above the graft union contained 0.28, 0.04, and 0.05 ng of PVS per milligram of tissue, respectively, as compared to an average of 12.93 ng of PVS per milligram of tissue in stem segment samples taken from just below the graft union from these same plants, or 18.17 ng of PVS per milligram of tissue in similar samples taken from plants containing nontransformed shoot grafts.

The cross-protection observed in grafts of PVM-infected tissue onto PVS-infected host plants was not reciprocal in that PVS-infected shoots grafted onto plants systemically infected with PVM contained similar titers of PVM, by 21 days following grafting, as noninfected shoots that had been similarly grafted (results not shown).

Susceptibility to infection with PVM and PVX. Transgenic plants expressing the PVS coat protein also showed a measure of resistance to infection by PVM following mechanical inoculation with purified virus. This resistance was not as complete as was observed following inoculation with PVS in that it could be overcome with high levels of inoculum and appeared to be related to the level of PVS coat protein expression. Transgenic RB41 potato,

Table 2. Accumulation of potato virus S (PVS) in tissue of nontransformed or transgenic potato plants following grafting onto transgenic or virus-infected host plants

Parent plant	Graft	DPI/DAG[a]	PVS concentration (ng/mg tissue)
RB41[b]	Nontransformed RB	42	≤0.017[c]
	RB41	42	≤0.026
Nontransformed RB[d]	None	42	15.98
PVS-infected RB[e]	Nontransformed RB	21	25.45
		42	32.33
	RB41	21	≤0.028
		42	0.265
	RB58	21	≤0.016
		42	0.083
	PVM-infected RB	21	≤0.019
		42	0.115
	None	...	23.93[f]

[a] Days postinoculation (DPI) or days after grafting (DAG).

[b] Shoots from either nontransformed or transgenic RB41 Russet Burbank potato plants were grafted onto transgenic RB41 parent plants. The concentration of PVS coat protein antigen present in the upper leaves of each graft was determined by double-antibody sandwich (DAS) ELISA of tissue samples removed 42 days after inoculation of the lower leaves of transgenic plants with a purified preparation of the Andean strain of PVS (2 μg/ml).

[c] Concentrations of PVS coat protein antigen in the range of ≤0.002–0.030 ng/mg of tissue represent the lower limit of detection by DAS-ELISA and are equal to the background values obtained from uninoculated transgenic plants.

[d] Concentration of PVS in the upper leaves of nontransformed control plants 42 days after inoculation with 2 μg/ml of PVS.

[e] Shoots from either nontransformed, transgenic RB41 or RB58 or from Russet Burbank potato plants systemically infected with potato virus M (PVM) were grafted onto PVS-infected parent plants that had been serially propagated as cuttings from previously infected plants. The level of PVS accumulation in the upper leaves of each graft was determined by DAS-ELISA 21 and 42 days after grafting.

[f] Average concentration of PVS in lower leaves of the systemically infected parent plant.

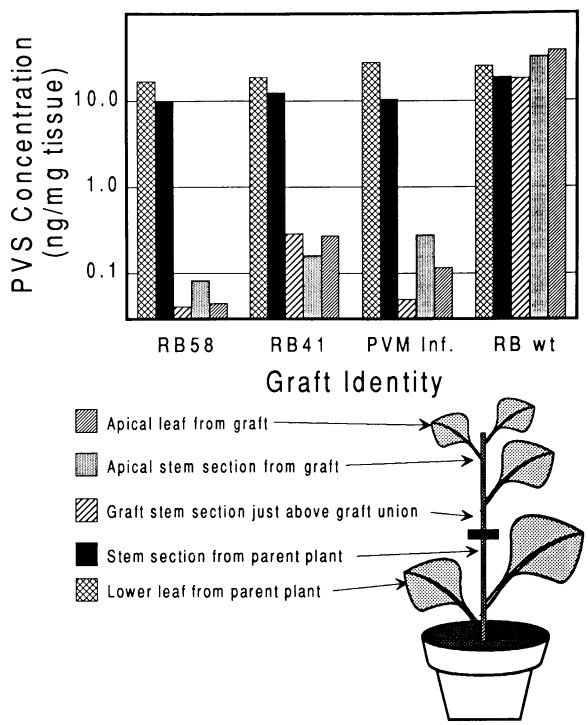

Fig. 4. Concentration of potato virus S (PVS) coat protein antigen in the tissues of systemically infected plants containing shoot grafts from transgenic plants (RB41 and RB58), plants infected with potato virus M (PVM), or nontransformed plants. Tissue samples from the lower infected leaves or stem segments of the parent plant, as well as stem segments from just above the graft union, and apical stem segments or apical leaves from the graft were removed 42 days after grafting and assayed for the presence of PVS by double-antibody sandwich ELISA. Viral coat protein concentrations are expressed as the $\log_{10}$ of nanograms of virus coat protein per milligram wet weight of tissue.

which expressed approximately twofold to fourfold higher levels of PVS coat protein than the RB52 line (Fig. 2), showed a lower titer of PVM (8.36 ng/mg of tissue) at 42 days following inoculation with 5 μg/ml of PVM than did similarly inoculated RB52 plants (25.2 ng/mg of tissue). Nontransformed control plants, which had been treated under the same conditions, showed more than a 10-fold higher concentration of PVM (91.8 ng/mg of tissue) in systemically infected leaves than did the transgenic RB41 plants (Fig. 5).

Not surprisingly, these transgenic plants failed to display any significant resistance to PVX infection and showed qualitatively similar symptom expression together with equal levels and rate of viral antigen accumulation as nontransformed control plants following inoculation with a severe strain of this virus. Also, transgenic plants inoculated with a mixture of PVX and PVS, while showing high titers of PVX (2–3 wk after inoculation), maintained their resistance to PVS infection (results not shown).

DISCUSSION

We have successfully introduced resistance to PVS into one of the most important commercial cultivars of potato, Russet Burbank. Transgenic plants, expressing the PVS coat protein, regenerated following transformation mediated by *A. tumefaciens*, were obtained with high frequency and were morphologically normal in appearance. The addition of Ag^+ to the shoot initiation medium had a profound effect on callus formation and proliferation with Russet Burbank leaves. It is known that potato plants *in vitro* can produce large amounts of ethylene, which is characterized by the formation of brown-yellow, friable callus which is nearly impossible to regenerate (Hussy and Stacey 1981). As previously reported by De Block (1988), callus formed in the presence of Ag^+ maintained a robust green color and was highly regenerable with multiple shoots initiating from each individual calli. This stimulatory effect of Ag^+ on callus formation has been suggested to be due to its ability to block ethylene action by binding to the ethylene receptor(s) (Aharoni *et al.* 1979; Beyer 1976).

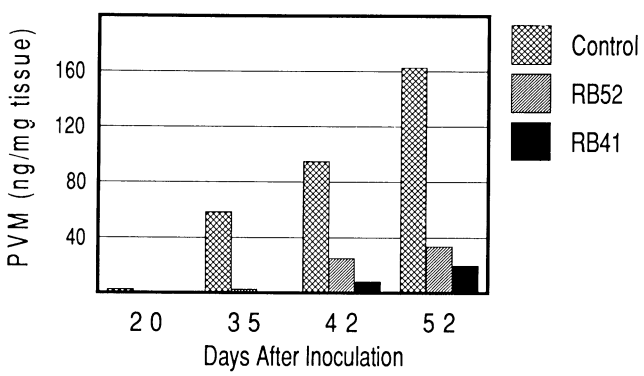

Fig. 5. Accumulation of potato virus M (PVM) coat protein antigen in the upper leaves of either nontransformed or RB52 and RB41 transgenic Russet Burbank potato plants after inoculation with intact PVM particles. Plants were mechanically inoculated with a purified preparation of PVM diluted to 5 μg/ml in 10 mM sodium phosphate buffer, pH 7.4. Viral coat protein concentrations were determined by double-antibody sandwich ELISA using a monoclonal antibody specific for the PVM capsid protein and are expressed as nanograms of virus coat protein per milligram wet weight of tissue.

Recent nucleotide sequence data from PVS (MacKenzie *et al.* 1989) and three related carlaviruses, PVM (Rupasov *et al.* 1989), lily symptomless virus (Memelink *et al.* 1990), and Helenium virus S (Foster *et al.* 1990), have shown that these viruses have genome organizations similar to that of PVX (Huisman *et al.* 1988) and other members of the potexvirus group. Despite differences in particle morphology, virus transmission, and the presence of an additional 3′ terminal ORF in the carlavirus genome, these data provide strong arguments for placing the carlaviruses and potexviruses in one taxonomic group.

Transgenic potato lines expressing PVS coat protein showed high levels of resistance to inoculation with intact PVS particles, and unlike previous studies with tobacco plants expressing TMV or alfalfa mosaic virus coat protein, these plants were also resistant to inoculation with PVS RNA. As we have previously reported (MacKenzie and Tremaine 1990), the fact that transgenic plants expressing PVS coat protein are protected from infection by PVS RNA, together with similar results obtained with plants expressing PVX coat protein (Hemenway *et al.* 1988), indicates that some event other than, or in addition to, virus uncoating is being inhibited.

The extent of this coat protein-mediated protection was further emphasized in plant grafting experiments in which nontransformed potato shoots grafted onto transgenic plants, which were subsequently inoculated with PVS, failed to accumulate any significant concentration of PVS. These results further indicate that inhibition of virus replication in primary infected cells also inhibits the systemic spread of the infectious moiety to other susceptible tissues.

Also, in reciprocal grafts, transgenic shoots remained essentially virus free by 21 days following grafting onto host plants systemically infected with PVS, and even after 42 days following grafting typically contained less than 2% of the PVS concentration of similarly grafted nontransformed shoots. This resistance to systemic infection by PVS was also mimicked in grafts of PVM-infected shoots onto PVS-infected plants. While it is likely that the long-distance systemic movement of PVS is via the phloem tissues of the vascular system, it is not known in what form the virus is transported. In the case of TMV it has been found that the TMV coat protein, but not necessarily virion assembly, is required for efficient long-distance spread (Oxelfelt 1975), and other studies have concluded that long-distance spread of TMV is via a viral ribonucleoprotein complex composed of several proteins including coat protein (Dorokhov *et al.* 1983, 1984). While not conclusive, the results presented here support the notion that long-distance transport of PVS may involve some form of the virus other than intact virions, since stem tissue taken from transgenic RB58 or RB41 grafts, just above the graft union, contained only 0.2 or 1.5%, respectively, of the level of PVS found in similar tissues from nontransgenic grafts. Alternatively, if PVS is in fact transported through the vascular tissue as intact particles, it must be present in exceedingly low concentrations, or possibly the transport of intact virions as well as virus replication may both be suppressed in transgenic phloem tissues. Wisniewski *et al.* (1990) have

recently described somewhat similar results using transgenic tobacco expressing the coat protein from TMV. In these experiments, grafts from plants expressing coat protein caused a delay in the movement of TMV from the inoculated nontransformed rootstock to similar nontransformed tissues above the graft union. While no conclusions regarding the nature of the transport form of TMV could be made, these results did support the hypothesis that the presence of coat protein in the phloem and associated cells interferes with the long-distance transport of TMV and systemic disease development.

Transgenic plants expressing the PVS capsid protein were also found to show a measure of resistance to infection with a related carlavirus from potato, PVM. The relative inefficiency of plants expressing PVS coat protein in inhibiting PVM infection was further emphasized by the observation that PVS-infected shoots also failed to significantly cross-protect against the systemic spread of PVM when grafted onto PVM-infected host plants, while PVM-infected shoots grafted onto plants systemically infected with PVS did inhibit the systemic spread of PVS into the graft. This type of nonreciprocal cross-protection is likely to account for the observation that infection with both of these viruses is often found under field conditions.

From nucleotide sequence analysis, the deduced amino acid sequences of the coat proteins from PVS and PVM are 44.7% identical, overall, and this level of homology increases to 73% if one examines a region that is 74 amino acid long beginning at position 180 of the PVS sequence. It is likely that the relatively high degree of homology between PVS and PVM in this region of the coat protein sequence is responsible for the partial resistance to PVM infection in plants expressing PVS coat protein. This central region of the coat protein sequence also contains significant blocks of homology with the coat protein sequences from a number of potexviruses, and may represent an internal domain involved in RNA-protein interactions during capsid assembly (MacKenzie *et al.* 1989).

The extent to which PVS-resistant transgenic potato cultivars retain their virus resistance properties under field conditions as well as the other intrinsically desirable characteristics of the Russet Burbank cultivar are currently being investigated.

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