Resistance in *Solanum brevidens* to both potato virus Y and potato virus X may be associated with slow cell-to-cell spread

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A series of experiments was carried out to investigate the nature of the resistance of the wild potato species, *Solanum brevidens*, to potato virus X (PVX) and potato virus Y (PVY). *In vitro* inoculation of leaf protoplasts of *S. brevidens* and the virus-susceptible dihaploid *S. tuberosum* genotype PDH40 with PVX or PVY using polyethylene glycol showed that protoplasts of both species were similar in susceptibility. However, examination of protoplasts prepared from the leaves of *S. tuberosum* and *S. brevidens* inoculated 2 to 5 weeks earlier showed that the percentage of PVX- and PVY-infected leaf cells of *S. tuberosum* were, respectively, 45- to 100-fold and about 1000-fold greater than the percentage of infected leaf cells of *S. brevidens*. These results suggest that resistance in *S. brevidens* to both PVX and PVY could be associated with slow cell-to-cell spread rather than with slow virus replication.

Introduction

Solanum brevidens Phil., a non-tuberizing diploid species, is resistant to potato leaf roll luteovirus (PLRV), potato Y potyvirus (PVY) and potato X potexvirus (PVX). For all these viruses, infection is systemic and resistance takes the form of a low virus titre in infected plants (Jones, 1979; Gibson *et al.*, 1990). This resistance has been transferred to the *S. tuberosum* gene pool by somatic hybridization (Austin *et al.*, 1985; Helgeson *et al.*, 1986; Fish *et al.*, 1988; Gibson *et al.*, 1988; Pehu *et al.*, 1989, 1990). Somatic hybrids (including aneuploids) between *S. brevidens* and PDH40, a dihaploid *S. tuberosum* genotype derived from cv. Pentland Crown and which is susceptible to PVX, PVY and PLRV (Fish *et al.*, 1988; Gibson *et al.*, 1988), mostly were resistant to PVX, PVY and PLRV or susceptible to all of them (Pehu *et al.*, 1990); the possibility of a common mechanism of resistance to PVX, PVY and PLRV in *S. brevidens* was suggested.

The mechanism of the virus resistance shown by *S. brevidens* has not been studied previously but the low virus titre suggested that either virus replication or virus movement was inhibited. To distinguish between these possibilities, we prepared protoplasts from leaves of plants infected with PVX or PVY and examined by an immunofluorescence assay whether all cells from *S. brevidens* had a low virus titre or whether most appeared uninfected and a few cells had a high titre. We also tested directly whether these viruses replicated readily in protoplasts of *S. brevidens* when infected *in vitro*.

Methods

Experimental plants. The test plants were clones of a dihaploid *S. tuberosum* line, PDH40 (De Maine, 1982) and *S. brevidens* (CPC 2451). Plants were maintained as *in vitro* shoot cultures on Murashige & Skoog (1962) medium (Flow Laboratories) at pH 5·8. The plants were chosen because they had been used in previous experiments with PVX and PVY (Gibson *et al.*, 1988, 1990; Pehu *et al.*, 1990). Their reaction to each virus seemed to be representative for each plant species and they had been used as parents in the production of somatic hybrids (Fish *et al.*, 1988; Jones, 1988). For all the experiments, plants were transplanted from *in vitro* shoot cultures to EFF compost (EFF Products), grown for 2 weeks in a propagator and then grown in a glasshouse with natural daylight extended to 18 h per day by artificial light.

Virus isolates, transmission and purification. One isolate each of PXV, PVY^O and PVY^N (Gibson *et al.*, 1990) were obtained from naturally infected potatoes and cultured in *Nicotiana tabacum* cv. White Burley for inoculation and purification. Experimental plants were dusted with carborundum abrasive and manually inoculated with infected tobacco sap.

Leaves were collected for the purification of PVX (Bercks, 1970; Pierpoint, 1973; McDonald *et al.*, 1976), PVY^O and PVY^N 21, 14 and 9

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days, respectively, after inoculation of tobacco plants. Viruses were partially purified by two cycles of low and high speed centrifugation. Finally, virus was resuspended in double-distilled water (PVX) or 0·01 M-borate buffer pH 7·5 (PVY); 0·05% sodium azide was added and the preparations were stored at 4 °C. The concentration of virus in each preparation was determined by electron microscopy.

Protoplast isolation and inoculation. Leaf protoplasts were isolated from *S. brevidens* and PDH40 using the methods of Nelson *et al.* (1983) and Foulger & Jones (1986), respectively, except that 1·5% cellulase R10 (Yakult Hansha) was used instead of meicelase for digesting leaves of *S. brevidens*. Protoplasts for inoculation with viruses were prepared from plants grown in a propagator in a glasshouse.

Protoplasts of *S. brevidens* and PDH40 were infected with PVX, PVY^O and PVY^N by a modified method based on that of Potrykus *et al.* (1987) using polyethylene glycol (PEG). Osmolarities of all solutions were adjusted with mannitol to 520 mOsM and 575 mOsM for *S. brevidens* and PDH40, respectively. Freshly purified virus preparations were dialysed against sterile distilled water (PVX) or 0·001 M-phosphate buffer, pH 7·5 (PVY) for 24 h and the osmolarity was adjusted to the above values just before use. Inoculation mixtures contained 50 µg of PVX or 100 µg of PVY and 5×10^5 protoplasts in 0·5 ml of 0·015 M-$MgCl_2$ solution, to which 40% (w/v) PEG 3350 (Sigma) in 0·1 M-$Ca(NO_3)_2$ pH 8·0 was added to give final PEG concentrations of 10, 15, 20 or 25%. Different PEG concentrations were used to ensure that optimum conditions for balance between membrane disruption for virus entry and subsequent protoplast viability were included. Protoplasts were incubated with virus for 30 min and then washed twice with protoplast wash solution (Nelson *et al.*, 1983). For inoculation with PVX, all the solutions, except PEG, were at pH 5·6. For inoculation with PVY, all the solutions were adjusted to pH 7·5, including the wash solution after inoculation. After washing, protoplasts were resuspended in culture solution (5·85 g V-KM culture medium salts made by Imperial Laboratories with 250 mg casein hydrolysate, 1 mg NAA, 0·4 mg BAP, 20 ml coconut milk, 81·4 g sucrose per litre) (Foulger & Jones, 1986) with 0·01% of the antibiotic cefotaxime (Roussel Laboratories) and cultured at 25 °C in the dark in an incubator.

Protoplasts were tested for presence of virus by an immunofluorescence assay (see later) 72 h after inoculation. The viability of the protoplasts was determined before and after inoculation and also before testing for virus by the proportion which fluoresced yellow-green after treatment with 0·01% fluorescein diacetate (FDA; Sigma) (Larkin, 1976).

The percentage of cells infected with PVX or PVY^N in leaves of infected *S. brevidens* and PDH40 plants was measured using protoplasts prepared from manually inoculated and systemically infected upper leaves; protoplasts from water-inoculated plants were also examined. Plants were inoculated within a week of transplanting them from culture. Plants of PDH40 were tested 2 weeks after inoculation with PVX or PVY^N, whereas plants of *S. brevidens* were tested 2 weeks after inoculation with PVX and 2 and 5 weeks after inoculation with PVY^N. Infected cells were detected using an immunofluorescence assay (see later). The experiment was repeated twice and four plants of both species were used in both experiments.

Serological assays for viruses. Polyclonal antisera to PVX and PVY were raised in rabbits by Dr D. A. Govier at Rothamsted, Hertfordshire, U.K. and antibodies were purified and conjugated with alkaline phosphatase (Clark & Adams, 1977). All antibodies and conjugated antibodies were diluted 1:500 before use, unless otherwise specified.

The titres of PVX or PVY in inoculated protoplasts, and the presence of PVX or PVY in plants, were examined by a double antibody sandwich ELISA using extraction buffer, phosphate-buffered saline (PBS), alkaline phosphatase-conjugated antibodies, nitrophenol phosphate as substrate and reading absorbances at 405 mn as described by Clark & Adams (1977). Protoplasts were tested by centrifuging a sample of 10^4 PVX-infected or 10^5 PVY-infected protoplasts from culture solution (5 min at 800 r.p.m.), removing the solution by pipette and grinding the protoplasts with sand in 250 µl of ELISA extraction buffer in a 1·5 ml Eppendorf tube. A further 250 µl of extraction buffer was added, followed by brief centrifugation in a microfuge to remove debris; samples were transferred into an antiserum-coated ELISA plate and assayed. The numbers of PVY particles in plants of *S. brevidens* and PDH40 inoculated with PVY were examined by immunosorbent electron microscopy (ISEM) (Roberts & Harrison, 1979).

The proportion of cells infected with PVX or PVY was examined by immunofluorescence assay. Microscope slides were washed in 90% ethanol, flamed and, when cool, covered with 100 µl 0·1% (w/v) poly-L-lysine (Sigma) and allowed to dry. Protoplasts in culture medium were spread gently over the slide and dried in a flow of warm air. Slides were put into distilled water for 4 to 6 h, then stained with a mixture containing anti-PVX or -PVY antiserum diluted 1:250 in PBS with 0·5% (w/v) bovine serum albumin (Sigma) and 1·5% sap from an uninfected plant, and incubated at 30 °C in a moist chamber for 2 h. They were then washed with three changes of PBS. Antiviral antibodies were detected by staining the slides with goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated goat antibodies (Sigma) diluted 1:250 in PBS and incubating at 5 °C in a moist chamber overnight. The slides were then washed as before, left to dry and covered with 50 µl of a solution containing three parts glycerol and one part 0·05 M-sodium carbonate buffer pH 9·6. After placing a coverglass on the slide, the number of infected protoplasts were scored by epifluorescence microscopy (Goding, 1976).

Results

Percentage of infected cells in leaves of PDH40 and S. brevidens *manually inoculated with PVX or* PVY^N

In protoplasts isolated from leaves of plants previously inoculated with PVX, the percentage of protoplasts identified as infected by immunofluorescence was about 50-fold greater for PDH40 than for *S. brevidens* (Table 1). The higher percentages observed in experiment B were after a warm sunny period that increased the temperature in the greenhouse. In *S. brevidens*, those protoplasts which fluoresced did so strongly and could be clearly distinguished from other protoplasts that sometimes exhibited green autofluorescence. This was particularly pronounced when the PVX-inoculated *S. brevidens* plants were tested 7 weeks after inoculation; 4% of the cells in the uppermost fully expanded leaves exhibited very bright fluorescence whereas other cells did not fluoresce at all.

In protoplasts prepared from leaves which themselves had been previously inoculated with PVY^N, the percentage of the protoplasts identified as infected by immunofluorescence in PDH40 was between 15 and 20% and many PVY particles were detected by ISEM, but neither infected protoplasts nor PVY particles were detected in *S. brevidens* (Table 1). However, 5 weeks after inoculation, between 0·01 and 0·1% of *S. brevidens* protoplasts

Table 1. *Percentages of protoplasts from PVX- or PVY^N-infected plants of PDH40 and* S. brevidens *which fluoresced following incubation with PVY or PVX antiserum and FITC conjugate*

Experiment	PDH40		*S. brevidens*	
	Expt. A	Expt. B	Expt. A	Expt. B
PVX				
Manually infected leaves	11* ± 0·9	87 ± 3·8	0·18 ± 0·04	2·1 ± 0·08
Systemically infected leaves	10 ± 0·3	85 ± 4·2	0·05 ± 0·02	1·9 ± 0·07
Uninoculated plants	0	0	0	0
PVY^N				
Manually infected leaves	21 ± 0·9	16 ± 1·3	0	0
			0·13 ± 0·01†	0·01 ± 0·01†
Systemically infected leaves	20 ± 0·8	15 ± 1·7	0	0
			0·07 ± 0·05†	0·08 ± 0·02†
Uninoculated plants	0	0	0	0
PVY particles/field‡	186 ± 10	145 ± 14	0	0
			0·09 ± 0·00†	0·1 ± 0·00†

* Mean percentages of infected protoplasts ± standard error. In each experiment 10000 protoplasts were scored.

† Values obtained 5 weeks after inoculation; all other values obtained 2 weeks after inoculation.

‡ Determined by ISEM of uninoculated leaves (magnification × 40000).

appeared to be infected and a few particles of PVY were seen by ISEM. No non-specific fluorescence was present.

Inoculation of protoplasts with PVX

Inoculation of protoplasts of PDH40 and *S. brevidens* directly with PVX using 20% PEG resulted in infection of about 30% of the protoplasts of both species (Table 2). *S. brevidens* protoplasts could tolerate a higher concentration of PEG (25%) than those of PDH40, as indicated by FDA staining, and 50% of them became infected when this higher concentration of PEG was used. Incubation in 20% PEG reduced the viability of PDH40 protoplasts by 10% and of *S. brevidens* protoplasts by 5%. Incubation in 25% PEG did not increase the mortality of *S. brevidens* protoplasts but possibly damaged them as the ELISA test indicated that the titre of PVX was less than that at 15% PEG, perhaps because the virus had replicated less (Table 2). In treatments where PVX and protoplasts were incubated without PEG, a few strongly fluorescing protoplasts were present that were apparently infected.

Inoculation of protoplasts with PVY

The highest levels of infections with PVY of protoplasts of PDH40 and *S. brevidens*, as judged by immunofluorescence microscopy, occurred using 10% and 15% PEG, respectively (Table 3). The proportions of infected protoplasts were generally lower than in PVX infection, being 5 to 10% with PVY^O for protoplasts of both species and 10 to 15% with PVY^N in *S. brevidens*. In PDH40 and *S. brevidens* protoplast preparations inoculated with PVY most protoplasts fluoresced to a limited extent, possibly because some PVY particles were bound to their plasma membranes, and only strong cytoplasmic fluorescence was considered to indicate infection. The ELISA absorbance values were similar for both PDH40 and *S. brevidens* protoplasts but direct comparison of the values was not possible because batches of protoplasts were assayed as different times.

Discussion

The titres of PVX and PVY in plants of *S. brevidens* have been reported to be 10-fold and 100-fold less, respectively, than the titres in PDH40 (Gibson *et al.*, 1990). In the present series of experiments, the proportion of leaf cells infected with PVX in *S. brevidens* was only 1 to 2% that of PDH40 2 weeks after inoculating leaves of whole plants of *S. brevidens* and PDH40 with PVX. Five weeks after inoculation with PVY^N a few infected leaf cells were found by immunofluorescence assay, whereas in PDH40 the number of leaf cells infected with PVY^N was usually more than 1000-fold greater than this just 2 weeks after inoculation. A few particles of PVY^N were found by ISEM in uninoculated leaves of infected plants of *S. brevidens* but as before, in PDH40 the numbers of PVY^N

Table 2. *Percentages of protoplasts inoculated with PVX which fluoresced after incubation with PVX antiserum and FITC conjugate*

	PDH40						*S. brevidens*					
	Expt. A		Expt. B		Expt. C		Expt. A		Expt. B		Expt. C	
PEG (%)	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2
25											51* (0·235)†	46 (0·219)
20			27	30	30 (0·261)	31 (0·258)	37	32	22	20		
15	23	20			21 (0·141)	23 (0·166)					28 (0·319)	23 (0·305)
10	10	13	24	28			4	6	14	17		
0	2	2	5	4	1 (0·083)	0 (0·071)	0	1	1	1	5 (0·112)	3 (0·068)
0 (no PVX)	0	0	0	0	0 (0·051)	0 (0·037)	0	0	0	0	0 (0·047)	0 (0·051)

* In each case 10000 protoplasts were scored.
† Figures in parentheses are A_{405} values generated in ELISA by 100000 protoplasts.

Table 3. *Percentages of protoplasts inoculated with PVY^O or PVY^N which fluoresced after incubation with PVY antiserum and FITC conjugate*

	PVY^O										PVY^N			
	PDH40				*S. brevidens*						*S. brevidens*			
	Expt. D		Expt. F		Expt. D		Expt. E		Expt. F		Expt. G		Expt. H	
PEG (%)	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2	Plant 1	Plant 2
25					5	9	7	5	6 (0·303)†	4 (0·290)	2	2	5 (0·365)	9 (0·951)
20	5	6	7 (0·347)	5 (0·299)										
15	5	7	11 (0·280)	9 (0·241)	11	12	10	13	1 (0·191)	3 (0·211)	11	9	15 (1·651)	13 (1·201)
10	10	12	11	11										
0	0	4	0	1	0	0	0	0·1	0 (0·080)	0 (0·064)	0	0	0 (0·358)	0 (0·271)
0 (no PVY)	0	0	0 (0·090)	0 (0·047)	0	0	0	0	0 (0·030)	0 (0·042)	0	0	0 (0·045)	0 (0·045)

* In each case 10000 protoplasts were scored.
† Figures in parentheses are A_{405} values generated in ELISA by 10000 protoplasts.

particles were more than 1000-fold greater. However, the experiments on the *in vitro* inoculation of leaf protoplasts of *S. brevidens* and PDH40 with PVX and PVY indicated that protoplasts of both species were similarly susceptible to PVX and PVY and that, given access, these viruses could replicate successfully in cells of either species.

These results suggest that the low titres of PVX and PVY in plants of *S. brevidens* and the small proportion of infected leaf cells are a result of restricted spread of these viruses and not a result of restricted replication. (The *in vitro* demonstration that both viruses can replicate successfully in *S. brevidens* cells is supported by the *in vivo* evidence that, in infected *S. brevidens* plants, the few cells that were fluorescent in the immunoassay fluoresced strongly, indicating a high virus titre; this evidence also suggests that the low virus titre in *S. brevidens* plants is probably not the result of inefficient inoculation or of a localized form of resistance, as a low virus titre in infected cells might be expected.) Both PVX and PVY were detected in uninoculated leaves of inoculated plants of *S. brevidens*, so only cell-to-cell and not long distance movement may be affected. This suggests that the infection was not subliminal (Cheo, 1970; Sulzinski &

Zaitlin, 1982; Hull, 1989). However, the distribution of the infected cells in leaves was not determined.

Two contrasting models have been developed to explain how viruses move from cell to cell in plants. There may be either a specific interaction between the virus or virus product and intercellular connections (plasmodesmata), or plasmodesmata might be non-specifically 'gated' open so that abnormally large molecules, including virions, viral nucleoproteins and certain dye molecules, can pass through (Fannin & Shaw, 1987; Meiners *et al.*, 1988; Wilson, 1988; Baulcombe & Hull, 1989). There is increasing evidence that 'gating' is facilitated by viral gene products, called movement proteins (Atabekov & Dorokhov, 1984; Meshi *et al.*, 1989; Lehto *et al.*, 1990). Coding sequences of the putative viral movement protein genes differ significantly and this implies that several different mechanisms may be utilized in cell-to-cell movement (Atabekov & Taliansky, 1990). However, the observation that distinctly different viruses can facilitate the spread of usually non-spreading viruses supports the idea that a common mechanism is utilized in cell-to-cell movement by many groups of viruses (Taliansky *et al.*, 1982; Meshi & Okada, 1986). Our results suggest that the resistance of *S. brevidens* to both PVX and PVY is associated with slow cell-to-cell movement, in support of the latter hypothesis. This also provides a means whereby the resistance of *S. brevidens* to PVX and PVY (and perhaps also PLRV; Gibson *et al.*, 1990) could have a common basis. The aim of future work will be to test the resistance of *S. brevidens* to a wider range of plant viruses. In addition, an asymmetric hybridization programme is in progress in which linkage group-specific restriction fragment length polymorphism will be used for long-range mapping of the resistance loci.

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