Transgenic *Nicotiana debneyii* expressing viral coat protein are resistant to potato virus S infection

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The coat protein gene from potato virus S (PVS) was introduced into *Nicotiana debneyii* by leaf disc transformation using *Agrobacterium tumefaciens*. Transgenic plants expressing the viral coat protein were highly resistant to subsequent infection by the ME strain of PVS as indicated by an absence of symptom development and a lack of accumulation of virus in both the inoculated and upper leaves. As in reported experiments with plants expressing potato virus X coat protein, plants expressing PVS coat protein were also protected from inoculation with PVS RNA. These results provide further evidence that coat protein-mediated protection for these two groups of viruses, which share similar genome organizations, may involve inhibition of some early event in infection other than or in addition to virus uncoating.

Potato virus S (PVS) is a member of the carlavirus group and is aphid-transmitted in a non-persistent manner to members of the *Solanaceae* and *Chenopodiaceae*. The viral RNA is encapsidated in an $M_0$ 33,000 coat protein into slightly flexuous filamentous particles which are 650 nm by 12 nm (Koenig, 1982). The viral genome consists of one single-stranded, positive sense RNA molecule with an estimated $M_0$ of $2.4 \times 10^6$ which contains a 3′-terminal polyadenylated region. Recent nucleotide sequence data from PVS (MacKenzie et al., 1989) and a related carlavirus, potato virus M (PVM) (Rupasov et al., 1989), have demonstrated that these two viruses have genome organizations similar to that of potato virus X (PVX) (Huisman et al., 1988) and other members of the potexvirus group. The viral genome of PVS contains six open reading frames (ORFs) which encode polypeptides with $M_0$ of 10734, 32515, 7222, 11802, 25092 and more than 41052. This latter ORF encodes amino acid sequences similar to those of putative viral replicase genes and the $M_0$ 32515 polypeptide has been shown to be the virus coat protein.

The first successful demonstration of genetically engineered resistance to a plant virus was reported by Powell-Abel and coworkers (1986) who found that transgenic tobacco plants expressing the coat protein gene from tobacco mosaic virus (TMV) displayed a significant delay in symptom development following inoculation with TMV. This form of coat protein-mediated protection has now been demonstrated for a number of different viruses including alfalfa mosaic virus (AlMV; Van Dun et al., 1987; Tumer et al., 1987; Loesch-Fries et al., 1987), cucumber mosaic virus (CMV; Cuozzo et al., 1988), tobacco rattle virus (Van Dun & Bol, 1988), tobacco streak virus (Van Dun et al., 1988) and PVX (Hemenway et al., 1988; Hoekema et al., 1989). More recently, simultaneous resistance to two viruses, PVX and potato virus Y, has been reported in transgenic potato plants expressing the nucleocapsid proteins from each of these viruses (Lawson et al., 1990).

We report here the production of transgenic *Nicotiana debneyii* which express the coat protein gene from PVS and are resistant to inoculation with PVS or purified viral RNA.

The preparation and characterization of a complementary DNA (cDNA) clone (pVS57), which contains the coding sequence of the entire PVS coat protein gene as an in-frame fusion with the lacZ α-peptide, has been previously described (MacKenzie et al., 1989). The insert contained in clone pVS57 corresponds to the 3′-terminal 1284 nucleotides from PVS RNA and the 5′ terminus of this insert is located 16 nucleotides upstream from the initiating ATG codon for the 33K viral coat protein gene. A *Hind*III fragment from pVS57 was inserted into *Hind*III-digested Bluescript vector and an appropriately oriented *EcoRI*-XhoI fragment from one of these resulting clones was subsequently transferred to an intermediate, co-integrate Ti plasmid vector, pCDX-1, which had been digested with *EcoRI* and XhoI. Vector pCDX-1 (Kay et al., 1987) is a derivative of pMON178 and contains a duplicated CaMV 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 *A. tumefaciens* strain GV3111SE.
Fig. 1. Immunoblot analysis of transgenic N. debneyii, line J3, expressing PVS coat protein. Immunoblots of coat protein (100 ng) from a purified preparation of PVS (lane 1) and total SDS-soluble protein (10 μg) extracted from J3 transgenic tissue (lane 2) or a non-transformed plant (lane 3) were incubated with MAb 1G11 followed by 125I-labelled goat anti-mouse Ig. The migration of PVS coat protein (CP) and Mr standards (×10^3) are indicated.

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 discs of N. debneyii were transformed using A. tumefaciens carrying the PVS coat protein constructs and shoots were regenerated according to the method of Horsch et al. (1985) on medium consisting of Murashige & Skoog salts, sucrose (30 g/l), benzyladenine (1·0 μg/ml), naphthalene acetic acid (0·1 μg/ml), kanamycin (250 μg/ml) and carbenicillin (500 μg/ml). Transformed shoots were subsequently rooted on phytohormone-free medium containing 100 μg/ml kanamycin and transferred to soil.

The ability of transgenic N. debneyii to produce PVS coat protein was assessed by immunoblotting using a specific monoclonal antibody 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 13000 r.p.m. for 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 Immobilon membranes (Millipore) and probed with 1G11 MAb and 125I-labelled goat anti-mouse Ig as previously described (MacKenzie et al., 1989). Approximately 40% of the transgenic plants obtained expressed detectable levels of PVS coat protein as judged by immunoblotting. The highest amounts of coat protein expressed as illustrated by line J3 (Fig. 1), were about 0·1% to 0·2% of total SDS-soluble protein.

Three transgenic lines (J3, J5 and J7) that expressed similar quantities of viral coat protein were used to evaluate resistance to PVS infection. The ME strain of PVS which infects N. debneyii was used in these tests as the Peruvian strain used in the cloning did not readily infect N. debneyii. The ME strain was purified as described previously (MacKenzie et al., 1989) from inoculated leaves of Chenopodium amaranticolor. Groups of F2 progeny plants (12 plants each group), which had been selected by germination on medium containing 200 mg/ml kanamycin, together with equal numbers of non-transformed N. debneyii, were mechanically inoculated with a preparation of purified virus at concentrations of 0·5, 1·0, 2·0 and 5·0 μg/ml. Other plants were inoculated with purified viral RNA (2 μg/ml) prepared from alkaline SDS-treated particles by multiple phenol–chloroform extractions and ethanol precipitation. The amount of viral antigen in the inoculated and upper leaves was measured 19, 27, 36 and 45 days after inoculation by using a double antibody sandwich (DAS) ELISA.

Leaf tissue samples (108 mg) were obtained using a no. 10 cork borer and processed through a mechanical leaf press irrigated with 0·5 ml of ELISA blocking buffer (10 mM-Tris·HCl pH 7·4, 0·15 M-NaCl, 1% bovine serum albumin, 0·05% Tween-20, 0·1% NaN3).

For DAS-ELISA, EIA microtitre plate wells were coated with 100 μl of purified rabbit anti-PVS Ig (20 μg/ml in 50 mM-sodium carbonate pH 9·6) overnight at 4 °C. Wells were then rinsed with water, treated with blocking buffer for 60 min and incubated with 100 μl volumes of serially diluted 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 Ig (200 ng/ml in blocking buffer; 90 min) and p-nitrophenyl phosphate substrate (0·5 mg/ml in 10% diethanolamine pH 9·8; 2 h at 24 °C). Concentrations of PVS in each sample were computed relative to a standard response curve constructed using samples of purified virus (5000 to 9·8 ng/ml) diluted in blocking buffer. Composite samples representing an average of at least four plants were analysed in triplicate for each time point and for each inoculation level. Control samples of non-inoculated transgenic plant tissue were also included.

After inoculation all of the control, non-transformed,
plants developed characteristic symptoms of vein clearing and chlorosis (Bagnall et al., 1959; Mackinnon & Bagnall, 1972) on the upper uninoculated leaves, with little or no apparent symptoms on the inoculated leaves. These symptoms were clearly discernible 3 weeks after inoculation with each of the inoculum concentrations tested. As indicated by DAS-ELISA (Fig. 2), these plants also had accumulated high levels of viral antigen in systemic leaves by 45 days after inoculation with concentrations ranging from 80 to 110 ng/mg wet weight of tissue. In contrast, the transgenic J3 plants neither exhibited symptoms nor accumulated greater than background levels of coat protein antigen after 45 days, even at the highest inoculum level tested (Fig. 2; Table 1). Transgenic lines J5 and J7, which expressed similar levels of viral coat protein to J3, were equally resistant to infection (Table 1).

In contrast to previous reports for plants expressing TMV or ALMV coat protein, inoculation of J3 transgenic plants with PVS RNA did not overcome protection (Table 1). This result is similar to those obtained using transgenic plants expressing PVX coat protein (Hemenway et al., 1988).

For many years it has been known that plants infected with mild strains of virus or viroids display a reduced or delayed symptom development when challenged with a second more virulent strain (McKinney, 1929; Fulton, 1986). Rose (1983) found that inoculation of N. debneyii with the symptomless CE strain of PVS could cross-protect against subsequent inoculation with PVS O which normally produces chlorosis of the upper leaves. It has been postulated that one of the underlying mechanisms of cross-protection is that the presence of coat protein from the first virus acts to inhibit the uncoating of the second challenge virus or that it encapsidates the RNA of the challenging strain thereby preventing its replication. Although results with viroids (Niblett et al., 1978) and coat protein-deficient mutants of TMV (Sarkar & Smitamana, 1981) argue against this mechanism, other experiments demonstrating that naked CMV RNA or TMV RNA, or TMV RNA trans-encapsidated in brome mosaic virus coat protein effectively escaped the protection afforded by previous infection with a mild strain of the relevant virus (Dodds et al., 1985; Sherwood & Fulton, 1982), indicate that inhibition of virus uncoating plays some role in cross-protection.

The extent to which classical cross-protection and the coat protein-mediated resistance in transgenic plants are related biological phenomena remains to be determined. It is likely that the mechanisms involved in each case are different and may in fact vary according to the particular virus involved. The fact that transgenic plants expressing PVS coat protein are protected from infection by PVS RNA, together with similar results obtained with PVX coat protein-expressing plants (Hemenway et al., 1988), indicates that some event other than or in addition to virus uncoating is being inhibited. In contrast to TMV, particle assembly of which proceeds in a 3' to 5' direction from an origin of assembly (OAS) located 925 nucleotides upstream of the 3' terminus of the RNA (Zimmern, 1977), the assembly of PVX is believed, by analogy with papaya mosaic virus (Lok & AbouHaidar, 1986), to occur in the 5' to 3' direction from an OAS located near the extreme 5' terminus of the RNA. It has been proposed by Hemenway et al. (1988) that PVX coat protein in transgenic plants may bind at or near this 5'-proximal OAS of the challenge viral RNA and subsequently

![Fig. 2. Accumulation of PVS coat protein antigen in the upper leaves of non-transformed N. debneyii (a) and J3 transgenic N. debneyii (b) after inoculation with PVS strain ME at concentrations of 0.5, 1.0 and 2.0 ng/μl at various days post-inoculation. The blank bar (c) shows the background value obtained with uninoculated J3 transgenic plants. Viral coat protein concentrations were determined by DAS-ELISA and are expressed as the log_{10} of ng virus coat protein/mg wet weight tissue.](image)

**Table 1. Concentration of PVS coat protein antigen in transgenic and normal Nicotiana debneyii 45 days after inoculation with either intact PVS or viral RNA**

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Inoculum level (μg/ml)</th>
<th>PVS concentration (ng/mg tissue)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inoculated leaf</td>
</tr>
<tr>
<td>J3 transgenic</td>
<td>0.5</td>
<td>≤0.1*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>≤0.2</td>
</tr>
<tr>
<td></td>
<td>2.0 (RNA)</td>
<td>ND†</td>
</tr>
<tr>
<td>J5 transgenic</td>
<td>2.0</td>
<td>≤0.2</td>
</tr>
<tr>
<td>J7 transgenic</td>
<td>2.0</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Non-transformed</td>
<td>0.5</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>2.0 (RNA)</td>
<td>ND</td>
</tr>
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</table>

* Concentrations of PVS coat protein antigen in the range of ≤0.1 to 0.2 ng/mg tissue represent the lower limit of detection by DAS-ELISA and are equal to the background values obtained from non-inoculated transgenic plants.

† ND, Not determined.
prevent translation of the replicase and/or interfere with replication. Such interactions may result in the at least partial recoating of infectious PVX RNA and would be consistent with the observation that TMV coat protein does not protect against inoculation with TMV RNA because, in the latter case, binding of coat protein subunits to the OAS site would not be expected to interfere with replicate translation or RNA replication, as indicated by cotranslational disassembly experiments (Wilson, 1984, 1986). The mechanism of disassembly/assembly of PVX is not known, but the extent to which PVX and PVX share similar genome organizations indicates that analogous processes may be involved.

Further studies on the mechanism of coat protein-mediated protection to PVS infection are currently in progress using transgenic lines of Ruset Burbank potato also engineered to express PVS coat protein.

We are indebted to Dr J. McPherson of the Department of Plant Science, University of British Columbia for providing the A. tumefaciens (GV3115sp/pTiB6535E) strain and the pCDX-1 vector. We also thank M. Elder, Vancouver Research Station, for providing the ME strain of PVS and W. MacDiarmid for photographic services.

References


(Received 28 February 1990; Accepted 22 May 1990)