

Movement protein-derived resistance to triple gene block-containing plant viruses

P. Seppänen,¹ R. Puska,¹ J. Honkanen,¹ L. G. Tyulkina,² O. Fedorkin,² S. Yu. Morozov² and J. G. Atabekov²

¹ Department of Biological Research, Kemira Agro OY, Espoo Research Centre, 02271 Espoo, Finland

² Department of Virology, Moscow State University, Moscow 119899, Russia

Two mutant potato virus X (PVX) movement protein (MP) genes (m12K-Sal and m12K-Kpn) were obtained by inserting specific linkers at the boundary between the N-terminal hydrophobic and putative transmembrane segment, and the central invariant hydrophilic region of the respective 12 kDa, 12K, triple gene block (TGB) protein. Several transgenic potato lines which expressed m12K-Sal or m12K-Kpn to different degrees were resistant to infection by PVX, potato aucuba mosaic potexvirus and the carlaviruses potato virus M and S over a wide range of inoculum concentrations (3–300 µg/ml). However, they were not resistant to potato virus Y, which lacks a TGB protein. We suggest that the resistance of m12K-Sal and m12K-Kpn transgenic potato lines is MP-derived and not RNA-mediated.

The efficiency of cell-to-cell virus movement is important in determining pathogenicity, virulence and, in some cases, the host range of a plant virus (reviewed by Atabekov & Taliansky, 1990; Maule, 1991). When the efficiency of the transport function and the rate of virus movement are reduced, the plant acquires a certain level of resistance to virus infection.

Production of dysfunctional or partially active movement proteins (MP) in transgenic plants is assumed to confer resistance to the wild-type (wt) virus by competition between wt virus-coded MP and the preformed modified MP (mMP). In support of this assumption, it has been reported that transgenic tobacco plants which produce a non-functional MP of tobacco mosaic virus (TMV) acquire resistance to TMV infection (Malyshenko *et al.*, 1993; Lapidot *et al.*, 1993). In addition, transgenic plants expressing non-functional TMV MP were resistant to several distantly related or unrelated viruses (Lapidot *et al.*, 1993; Cooper *et al.*, 1995). These results suggest

that there are conserved functions among the MP of different plant viruses, despite an apparent lack of sequence similarity.

Unlike tobamoviruses and many other viruses which have a single MP gene, a module of three partially overlapping genes known as the triple gene block (TGB) was found in the genomes of potexviruses, carlaviruses, hordeiviruses and some furo-like viruses (Morozov *et al.*, 1989; Rupasov *et al.*, 1989; Scott *et al.*, 1994). Mutations in each of the TGB genes have been shown to inhibit the virus transport function (Petty & Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Angell *et al.*, 1996). The first ORF of the potato virus X (PVX) TGB encodes a protein of molecular mass 25 kDa (25K) (Fig. 1*a*; Skryabin *et al.*, 1988). The 12K and 8K proteins encoded by the second and third ORFs of PVX TGB (Fig. 1*a*), and their counterparts in other viruses, possess hydrophobic domains (Fig. 1*b*) and were found to associate with a membrane-enriched fraction *in vitro* and *in vivo* (Morozov *et al.*, 1990; Richards & Tamada, 1992; Donald *et al.*, 1995).

Recently, it was shown that *Nicotiana benthamiana* plants which express a modified 13K MP, encoded by the central TGB ORF of white clover mosaic potexvirus (WCIMV) (Fig. 1*b*), were resistant to systemic infection with WCIMV, two other potexviruses and potato virus S (PVS) (Beck *et al.*, 1994). In this paper, we show that expression in transgenic potato plants of the PVX 12K MP gene, specifically modified at the region encoding the boundary between the hydrophobic and hydrophilic segments, confers MP-derived resistance to PVX, potato aucuba mosaic potexvirus (PAMV) and the carlaviruses potato virus M (PVM) and PVS. Movement of these viruses within the inoculated leaves was blocked. However, the plants were susceptible to potato virus Y (PVY).

A portion of the PVX genome including the 12K and 8K genes (Fig. 1*a*) was amplified and cloned into the T7-transcription vector pBS (Stratagene) to give pXT7-12, as described (Morozov *et al.*, 1991). pRT12WT was constructed by cutting pXT7-12 at an *EcoRI* site, artificially introduced upstream of the 12K gene, and a *HincII* site (position 5685 in the PVX genome) and inserting the isolated fragment (containing the wt TGB 12K and 8K protein genes) into *EcoRI*–*SmaI*-cleaved pRT101 (Topfer *et al.*, 1987). pUC12WT

Author for correspondence: Joseph Atabekov.

Fax + 7 095 938 06 01. e-mail atabekov@virus.genebee.msu.su

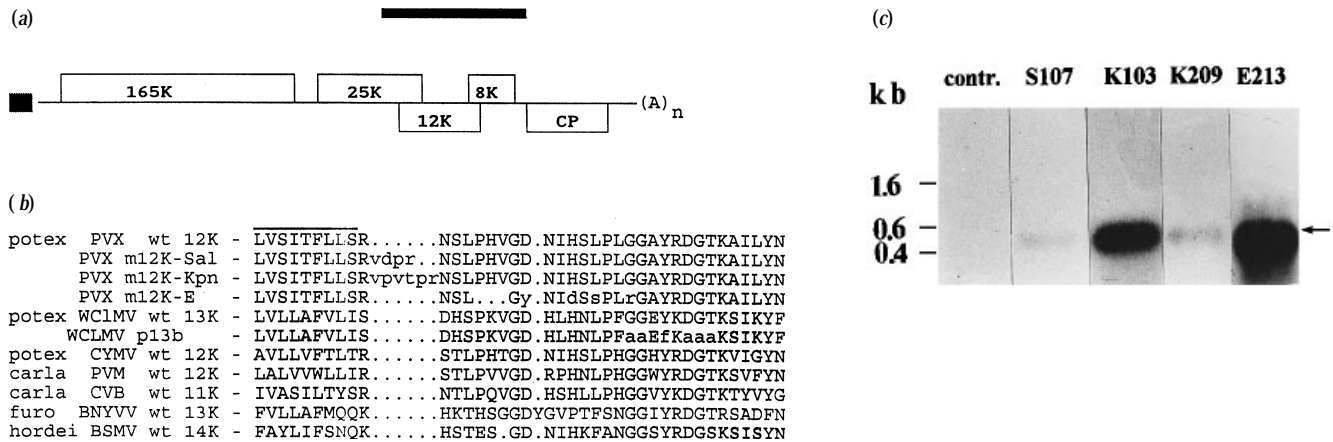


Fig. 1. (a) Schematic representation of the PVX genome. Non-coding regions are shown as a single line. Coding regions are shown by open boxes and indicated by the molecular mass (K, kDa) of their products or as coat protein (CP). The filled box indicates the 5' cap structure. The thick line above shows the portion of the PVX genome cloned in the transgenes of the S-, K- and E-series. (b) Alignment of conserved hydrophilic regions of the proteins coded by the central TGB ORF of PVX, white clover mosaic (WCLMV) and clover yellow mosaic (CYMV) potexviruses, potato virus M (PVM) and chrysanthemum virus B (CVB) carlaviruses, beet necrotic yellow vein furovirus (BNYVV) and barley stripe mosaic hordeivirus (BSMV). The positions of amino acid substitutions in the 12K MP of the WCLMV mutant p13b (Beck *et al.*, 1994) and the PVX mutant m12K-E, and the insertions in PVX mutants m12K-Sal and m12K-Kpn are shown in lower-case letters. Part of the N-terminal hydrophobic segment is overlaid above the PVX sequence. (c) Northern blot analysis of total RNA extracted from leaves of untransformed potato (cv. Pito) as a control and mMP⁺ transgenic lines S107, K103, K209 and E213. Molecular masses are marked in kb. The arrow indicates the predicted position of 12K/8K mRNA expressed from integrated chimaeric pBIN sequences. The probe was ³²P-labelled pUC12WT.

was constructed by subcloning a *XhoI*–*Bam*HI fragment from pRT12WT into *SalI*–*Bam*HI-cleaved pUC19.

To create the m12K-Sal and m12K-Kpn mutants of the 12K MP several codons were inserted (in frame) into the central hydrophilic domain of the 12K protein gene (Fig. 1*b*). A 12 nt insertion (mutant m12K-Sal) and an 18 nt insertion (mutant m12K-Kpn) in the 12K protein gene were created by inserting a modified *SalI* linker (octamer) and a *KpnI* linker (14-mer), respectively, into the filled *XbaI* site (position 5249 in the PVX genome) of pUC12WT, to give pUC-Sal and pUC-Kpn. pRT-Sal and pRT-Kpn were constructed by inserting an *EcoRI*–*Bam*HI fragment of pUC-Sal and pUC-Kpn, respectively, into pRT101.

The mutant m12K-E (Fig. 1*b*) used in this study was constructed by PCR-mediated mutagenesis using pUC12WT DNA as template and two primers: 12mod-*Xba*, 5' GCTTctagaAATAGTTTtaggTTATAACATTGACAGTTTcaccACACAGAGGA 3', and the universal sequencing primer. The primer 12mod-*Xba* corresponds to an internal region of the PVX 12K gene including an authentic *XbaI* site (shown in lower-case) and a 9 nt deletion and four single base pair changes compared to the wild-type PVX RNA sequence (positions 5245–5305). The resulting PCR fragment was cleaved with *XbaI* and *Bam*HI and cloned into pUC12WT, to give pUC-E. pRT-E was constructed by inserting the *EcoRI*–*Bam*HI fragment of pUC-E into pRT101. The 12K gene in pUC-E was sequenced to confirm the modified gene. Plant expression cassettes were constructed in the binary vector pBIN19 (Bevan, 1984) by excision of *Hind*III fragments from

the pRT-based plasmids (pRT-Sal, pRT-Kpn and pRT-E) and insertion into *Hind*III-cut pBIN19.

pBIN19 plasmids containing the m12K-Sal, m12K-Kpn or m12K-E genes were conjugated into *Agrobacterium tumefaciens* pGV2260, a non-oncogenic helper strain. Pieces of potato stem (*Solanum tuberosum* cv. Pito) were used for *Agrobacterium*-mediated transformation as described by Truve *et al.* (1993). Transgenic lines with the modified m12K-Sal, m12K-Kpn and m12K-E genes were referred to as S-, K- and E-series lines, respectively.

Of 44 transformed potato lines 15 S-, 12 K- and 10 E-series lines were selected for further analyses by Northern and/or Southern blotting. In Northern blots, several independent lines of each series showed the presence of 12K and 8K mRNA (mMP⁺) of the expected size. Fig. 1(*c*) shows Northern blot analyses of some lines used for further testing of virus resistance. Some lines showed no 12K or 8K RNA expression (data not shown) and were not tested for resistance. Significant differences in RNA expression levels were also noticed between different mMP⁺ clones (Fig. 1*c* and data not shown).

Three-week-old plants obtained from transformed tissue explants, with five to six true leaves and already containing leaflets, were used for challenge virus inoculations (three to six leaves were inoculated per plant). Three inoculum dilutions (300, 30 and 3 µg/ml) of PVX (Russian strain) were used for the infectivity tests. Leaf tips (about one-third of the leaf), were inoculated mechanically with 20–30 µl inoculum. Virus accumulation was tested by DAS-ELISA (Clark & Adams, 1977) 10 days post-inoculation (p.i.) in apical inoculated (AI), non-

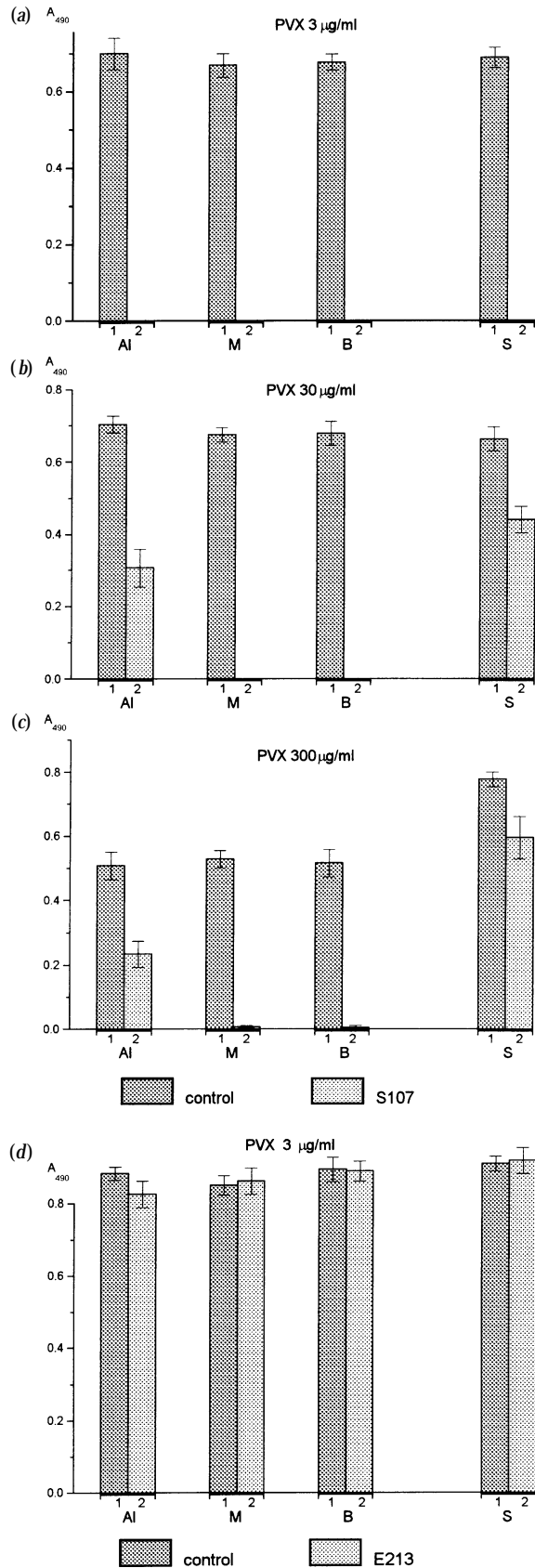


Fig. 2. Reaction of potato plants expressing the modified 12K MP PVX

inoculated middle (M) and basal (B) sections of the challenged leaf, as well as in non-inoculated (systemically infected) leaves (S) at 30 days p.i. All leaf sections from one plant were pooled for further testing by DAS-ELISA.

To check for virus resistance, selected lines with different levels of PVX-specific mRNA expression (Fig. 1c) in the S- (S101, S102, S107, S113, S115 and S119), K- (K103, K106, K107, K207, K209 and K210) and E-series (E203, E204, E213 and E215) plants, as well as non-transgenic control plants, were inoculated with PVX and other viruses. Plants of line S107 (Fig. 2), K209 (Fig. 3d), K103, K106, K107, K207, S101, S102, S113 and S115 (data not shown), which expressed modified 12K genes (mMP⁺), were completely resistant to PVX infection at an inoculum concentration of 3 µg/ml. Consequently, both cell-to-cell and systemic movement of PVX were blocked in transgenic mMP⁺ plants of the K- and S-series. Importantly, there was no correlation between 12K transgene mRNA accumulation and resistance (that is high levels of resistance were found both in high and low mRNA expressing lines). We propose that even low level m12K expression supports the resistant phenotype.

At higher PVX inoculum concentrations (30–300 µg/ml), the accumulation of PVX in AI leaf sections of mMP⁺ plants was reduced in comparison to control MP⁻ (Fig. 2b, c); however, the level of resistance was considerably lower than that with an inoculum concentration of 3 µg/ml. It should be noted that when virus accumulated to a certain level in the AI section of the leaf, systemic spread of infection occurred even in the absence of virus accumulation in the M and B sections (Fig. 2b, c).

mMP⁺ plants of the m12K-E series were exceptional. Despite the high level of PVX-specific RNA expression detected by Northern blotting, no resistance to PVX infection was detected in all four lines tested: E213 (Fig. 2d), E203, E204 and E215 (data not shown). The reason for the inability of this 12K MP modification to confer resistance to PVX is not clear.

PAMV potexvirus is distantly related to PVX, whereas PVS and PVM are members of the genus *Carlavirus*. Although these viruses encode TGB proteins, their genomes share little nucleotide sequence similarity with the PVX genome (Xu *et al.*, 1994; Rupasov *et al.*, 1989). Transgenic potato plants (mMP⁺ line K209) transformed with the mutated PVX 12K and wt 8K

gene to PVX. (a–c) Resistance of plants transgenic for the m12K-Sal gene (line S107) to PVX at three inoculum concentrations (3, 30 or 300 µg/ml). Analogous data (not shown) were obtained with the m12K-Kpn gene. (d) Susceptibility of plants transgenic for the m12K-E gene (line E213) to PVX inoculated at 3 µg/ml. A₄₉₀ represents the DAS-ELISA absorbance values with peroxidase-conjugated antibodies. Virus was detected by DAS-ELISA in pooled samples from five to six inoculated leaves of each plant and data were averaged for five to six separate plants 10 days p.i. (for inoculated leaves) and 30 days p.i. (for systemic leaves). Standard values with peroxidase-conjugated antibodies; M, middle non-inoculated; B, basal non-inoculated sections of the same leaf; S, systemically infected leaves. Columns 1, control (MP⁻) plants; Columns 2, Mmp⁺ transgenics of lines S107 (a–c) or E213 (d) expressing a mutated 12K MP gene.

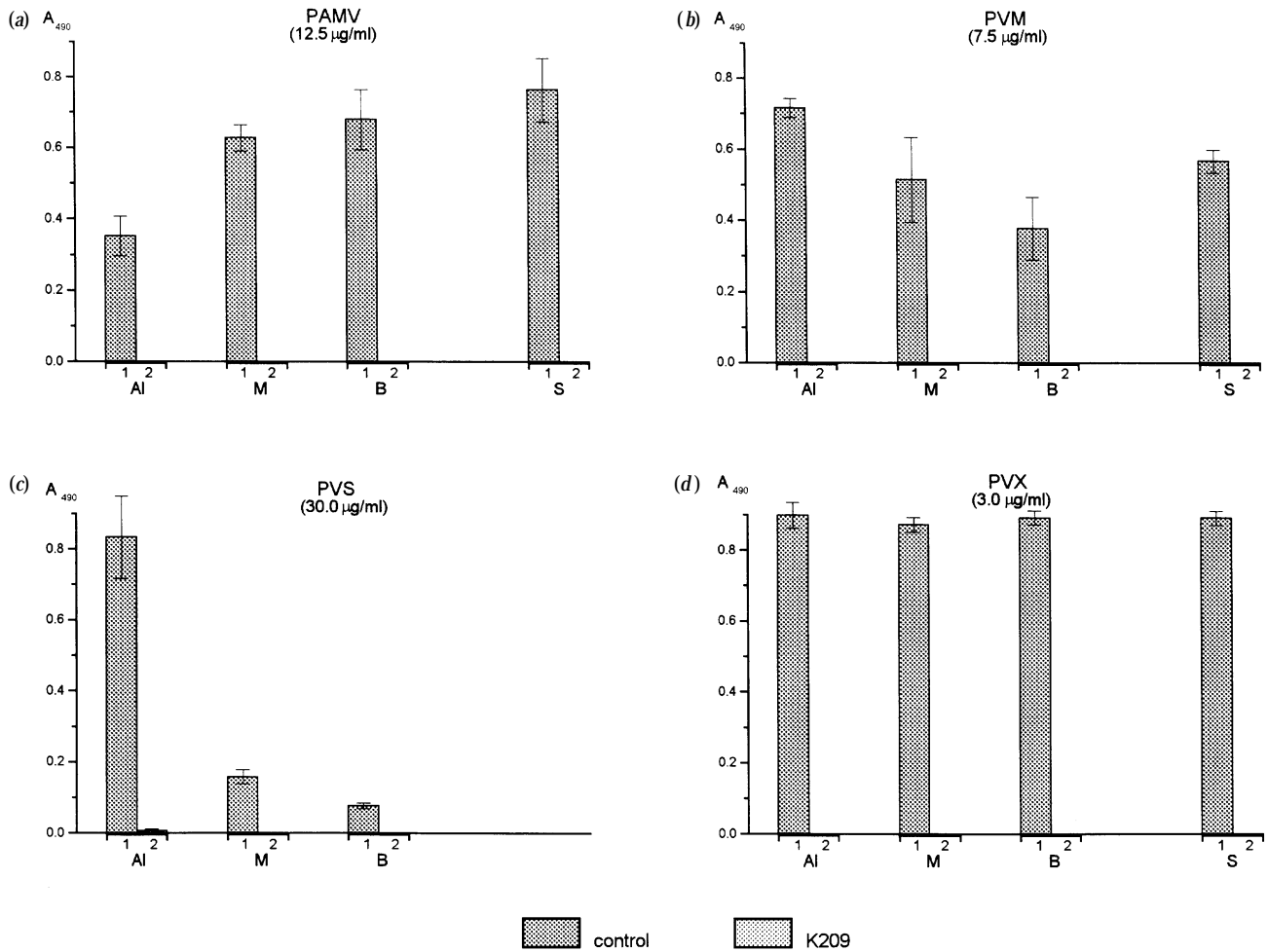


Fig. 3. Resistance of potato plants expressing a modified 12K MP PVX gene (line K209) to (a) PAMV, (b) PVM, (c) PVS, (d) PVX. Bars show DAS-ELISA data for the accumulation of each virus in transgenic PVX mMP⁺ plants or control plants inoculated with 12.5 µg/ml PAMV, 7.5 µg/ml PVM, 30 µg/ml PVS or 3 µg/ml PVX, respectively. Abbreviations are as for Fig. 2. Columns 1, control (MP⁻) plants; Columns 2, Mmp⁺ transgenics of line K209 expressing a mutated 12K MP gene.

genes were inoculated with PAMV, PVS and PVM by the procedure described above. Fig. 3(a-c) shows that transgenic plants were resistant to all three viruses, at least at the inoculum concentrations used in our experiments.

It can be concluded that both cell-to-cell movement and systemic spread of PAMV, PVS and PVM were blocked in the K209 transgenic plants. In a separate series of experiments (data not shown), we found that transgenic plants of lines K103 and S107 were also resistant to PAMV, PVS and PVM.

To determine whether the resistance was also manifest against viruses lacking a TGB gene, transgenic K- and S-series potato plants were inoculated with PVY⁰. No significant differences in the levels of PVY⁰ accumulation in inoculated or in systemically infected leaves were observed in mMP⁺ transgenic plants compared with control (MP⁻) potato plants (data not shown).

Transgenic mMP⁺ *N. benthamiana* plants (Beck *et al.*, 1994) which expressed the mutated 13K WCIMV MP (Fig. 1b) were found to be resistant to systemic infection with WCIMV,

PVX, PVS and narcissus mosaic potexviruses, but were susceptible to TMV, lacking a TGB protein. These observations suggested that expression of the modified 12K/13K MP gene of a TGB was able to protect transgenic plants against a range of viruses possessing a TGB protein; however, there was no protection against viruses lacking a TGB protein.

We suggest that the resistance of mMP⁺ plants (S- and K-series) was due to interference between the modified (presumably partially functional) 12K MP and that of the challenging virus, rather than to a sense-RNA-mediated effect (Smith *et al.*, 1994; English *et al.*, 1996). Unfortunately, this particular point was not discussed by Beck *et al.* (1994).

Several lines of evidence strongly support this theory. Firstly, the susceptibility of four lines of E-series plants to PVX (Fig. 2d) shows that, despite the almost complete sequence homology between wt PVX RNA and the RNA produced by the m12K-E transgene, there was no resistance to PVX. This point is also supported by our unpublished data on the complete lack of resistance to PVX in *N. benthamiana* and *N.*

tabacum plants expressing wt PVX 12K and 8K protein genes. Secondly, a high level of PVX accumulation (over 70% in comparison with wt plants) in non-inoculated systemic leaves of transgenic potato (Fig. 2*b, c*) is not consistent with the blockage of RNA replication or 'recovery' phenotype provoked by an RNA-mediated mechanism. Finally, it is unlikely that resistance of mMP⁺ plants to four different viruses (PVX, PAMV, PVS and PVM) with low nucleotide sequence identity (30–50%) in the TGB region was caused by the highly sequence-specific nature of RNA-mediated resistance (English *et al.*, 1996). Additionally, in the most conserved hydrophilic region, PVX displays 68–70% amino acid sequence identity and only 62–63% nucleotide sequence identity with PAMV, PVS and PVM. Also, in the m12K-E transgene there is a \geq 90% nucleotide sequence identity to wt in the hydrophilic region yet no resistance was observed in plants of four independent lines. These facts unequivocally argue against an RNA-mediated mechanism of resistance. It is probable that the MP encoded by the central gene of the TGB of different viruses share a common function not present in the MP of TGB-lacking viruses. Therefore, the modified 12K genes (m12K-Sal and m12K-Kpn) act in mMP⁺ plants as dominant negative mutants affecting different TGB-possessing viruses.

We thank Dr A. F. Bobkova for DAS-ELISA tests and Dr A. G. Solovyev for some gene constructs. We also thank Dr T. Teeri for the *Agrobacterium* vector constructs and K. Koivunen, A. Kerttula and A. Aaltonen for transformation and technical assistance.

References

- Angell, C. H., Davies, C. & Baulcombe, D. C. (1996). Cell-to-cell movement of potato virus X is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of *Nicotiana clevelandii*. *Virology* **216**, 197–201.
- Atabekov, J. G. & Taliansky, M. E. (1990). Expression of a plant virus-coded transport function by different viral genomes. *Advances in Virus Research* **38**, 201–247.
- Beck, D. L., Guilford, P. J., Voot, D. M., Andersen, M. T. & Forster, R. L. S. (1991). Triple block proteins of clover mosaic potexvirus are required for transport. *Virology* **183**, 695–702.
- Beck, D. L., Van Dolleweerd, C. J., Lough, T. J., Balmori, E., Voot, D. M., Andersen, M. T., O'Brien, I. E. W. & Forster, R. L. S. (1994). Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block. *Proceedings of the National Academy of Sciences, USA* **91**, 10310–10314.
- Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12**, 8711–8721.
- Clark, M. F. & Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475–483.
- Cooper, B., Lapidot, M., Heick, J. A., Dodds, J. A. & Beachy, R. N. (1995). A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility. *Virology* **206**, 307–313.
- Donald, R. G. K., Petty, I. T. D., Zhou, H. & Jackson, A. O. (1995). Properties of genes influencing barley stripe mosaic virus movement phenotypes. In *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance. Proceedings of the Fifth International Symposium*, pp. 115–150. Edited by D. D. Bills and others. Singapore: World Scientific.
- English, J. J., Mueller, E. & Baulcombe, D. C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 179–188.
- Gilmer, D., Bouzoubaa, S., Hein, A., Guilley, H., Richards, K. & Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. *Virology* **189**, 40–47.
- Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J. & Beachy, R. N. (1993). A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant Journal* **4**, 959–970.
- Malysenko, S. I., Kondakova, O. A., Nazarova, Ju. V., Kaplan, I. B., Taliansky, M. E. & Atabekov, J. G. (1993). Reduction of tobacco mosaic virus accumulation in transgenic plants producing non-functional viral transport proteins. *Journal of General Virology* **74**, 1149–1156.
- Maule, A. (1991). Virus movement in infected plants. *Critical Reviews in Plant Sciences* **9**, 457–473.
- Morozov, S. Yu., Dolja, V. V. & Atabekov, J. G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *Journal of Molecular Evolution* **29**, 52–62.
- Morozov, S. Yu., Miroshnichenko, N. A., Zelenina, D. A., Fedorkin, O. N., Solovyev, A. G., Lukasheva, L. I. & Atabekov, J. G. (1990). Expression of RNA transcripts of potato virus X full-length and subgenomic RNAs. *Biochimie* **72**, 677–684.
- Morozov, S. Yu., Miroshnichenko, N. A., Fedorkin, O. N., Solovyev, A. G., Zelenina, D. A., Lukasheva, L. I., Karasev, A. V., Dolja, V. V. & Atabekov, J. G. (1991). Expression strategy of the potato virus X triple gene block. *Journal of General Virology* **72**, 2039–2042.
- Petty, I. T. D. & Jackson, A. O. (1990). Mutational analysis of barley stripe mosaic virus RNA β . *Virology* **179**, 712–718.
- Richards, K. E. & Tamada, T. (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annual Review of Phytopathology* **30**, 291–313.
- Rupasov, V. V., Morozov, S. Yu., Kanyuka, K. V. & Zavriev, S. K. (1989). Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. *Journal of General Virology* **70**, 1861–1869.
- Scott, K. P., Kashiwazaki, S., Reavy, B. & Harrison, B. D. (1994). The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. *Journal of General Virology* **75**, 3561–3568.
- Skryabin, K. G., Morozov, S. Yu., Kraev, A. S., Rozanov, M. N., Chernov, B. K., Lukasheva, L. I. & Atabekov, J. G. (1988). Conserved and variable elements in RNA genomes of potexviruses. *FEBS Letters* **240**, 33–40.
- Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A. & Dougherty, W. G. (1994). Transgenic plant virus resistance mediated by untranslated sense RNAs: expression, regulation and fate of nonessential RNAs. *Plant Cell* **8**, 1444–1453.
- Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. & Steinbiss, H.-H. (1987). A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Research* **15**, 5890.
- Truve, E., Aaspollu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T., Kelve, M., Seppänen, P. & Saarma, M. (1993). Transgenic

potato plants expressing mammalian 2'-5' oligoadenylate synthetase are protected from potato virus X infection under field conditions. *Bio/Technology* **11**, 1048-1052.

Xu, H., Leclerc, D., Leung, B. & AbouHaidar, M. G. (1994). The entire

nucleotide sequence and genomic organization of potato aucuba mosaic potexvirus. *Archives of Virology* **135**, 461-469.

Received 21 October 1996; Accepted 14 January 1997