Short communication

Potato virus X movement in *Nicotiana benthamiana*: new details revealed by chimeric coat protein variants

CAMILLA BETTI^{1,†,‡}, CHIARA LICO^{1,†}, DARIO MAFFI², SIMONE D'ANGELI³, MARIA MADDALENA ALTAMURA³, EUGENIO BENVENUTO¹, FRANCO FAORO^{2,4,*} AND SELENE BASCHIERI^{1,*}

¹Unità Tecnica Biologia delle Radiazioni e Salute dell'Uomo, Laboratorio di Biotecnologie, ENEA C.R. Casaccia, Via Anguillarese 301, 00123, Rome, Italy

²Dipartimento di Produzione Vegetale, Università degli Studi di Milano, Via Celoria 2, 20133, Milan, Italy

³Dipartimento di Biologia Vegetale, Università 'Sapienza' di Roma, P.le A. Moro 5, 00185, Rome, Italy

⁴CNR, Istituto di Virologia Vegetale U.O. Milano, Via Celoria 2, 20133, Milan, Italy

SUMMARY

Potato virus X coat protein is necessary for both cell-to-cell and phloem transfer, but it has not been clarified definitively whether it is needed in both movement phases solely as a component of the assembled particles or also of differently structured ribonucleoprotein complexes. To clarify this issue, we studied the infection progression of a mutant carrying an N-terminal deletion of the coat protein, which was used to construct chimeric virus particles displaying peptides selectively affecting phloem transfer or cell-to-cell movement. *Nicotiana benthamiana* plants inoculated with expression vectors encoding the wild-type, mutant and chimeric viral genomes were examined by microscopy techniques. These experiments showed that coat protein–peptide fusions promoting cell-to-cell transfer only were not competent for virion assembly, whereas long-distance movement was possible only for coat proteins compatible with virus particle formation. Moreover, the ability of the assembled PVX to enter and persist into developing xylem elements was revealed here for the first time.

Potato virus X (PVX) flexible filamentous virions consist of a single-stranded, positive-sense, RNA molecule, encapsidated by approximately 1300 copies of the coat protein (CP). Virus particles have a modal length of 500 nm and a diameter of 13 nm. The genome encodes five multifunctional proteins: the viral replicase (an RNA-dependent RNA polymerase, RdRP), the triple gene block (TGB) movement proteins (TGBp1, TGBp2 and TGBp3) and the CP (Atabekov *et al.*, 2007).

The model outlining the PVX infection mechanism is becoming more and more detailed, particularly the role of TGB proteins (Ju

et al., 2007, 2008; Mekuria *et al.*, 2008; Samuels *et al.*, 2007). However, whether the contribution of CP to cell-to-cell movement is strictly dependent on virion assembly has not been clearly established to date (Verchot-Lubicz *et al.*, 2010). One model supports the concept that virions are necessary for transfer from infected to uninfected cells via plasmodesmata (PD) (Santa Cruz *et al.*, 1998); another suggests that intercellular transfer is mediated by a differently structured ribonucleoprotein (RNP) complex, including CP and TGBp1 (Lough *et al.*, 2000). Recently, *in vitro* studies have hypothesized that viral RNA can move through PD both as an RNP complex and as fully assembled virions associated with TGBp1 (Karpova *et al.*, 2006; Lukashina *et al.*, 2009; Zayakina *et al.*, 2008).

To gain knowledge on the PVX infection process and, in particular, on the role of CP, the tissues of plants inoculated with viral expression vectors encoding mutated and chimeric PVX particles (originally developed to formulate peptide vaccines and affected in different movement phases) were studied by different microscopic approaches. The isolation and behaviour of a mutant lacking the N-terminal 21 amino acids of the CP (PVX*Sma*) have been analysed in previous studies, revealing the effects produced on viral movement by fusion to the mutated CP of a panel of immune epitopes differing in amino acid composition and length (Lico *et al.*, 2006, 2009). In these studies, it was concluded that: (i) the generation of infectious chimeric virus particles is strongly influenced by the isoelectric point (pI) and the tryptophan content of the inserted peptides; and (ii) the genetic stability and preservation of the correct amino acid sequence fused at the CP N-terminus depend strictly on the presence of serine and threonine residues in the peptide.

The effects of fusion to PVX*Sma* CP of four peptides (Z13, p18, p18DD and NP; Table 1) on virus particle assembly and movement have been studied here at the microscopic and ultrastructural levels. This approach was preferred to the classical use of green fluorescent protein (GFP) (Baulcombe *et al.*, 1995; Santa Cruz *et al.*, 1996) because free cytosolic GFP can move through PD by passive diffusion (Schönknecht *et al.*, 2008).

*Correspondence: Email: selene.baschieri@enea.it; franco.faoro@unimi.it

†These authors contributed equally to this work.

‡Present address: Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, B-9052 Gent, Belgium.

Table 1 N-terminal portions of the coat proteins (CPs) encoded by the wild-type, mutant and chimeric constructs and the respective infection phenotypes.

Alignment of the N-terminal portion of the encoded CP	Construct name	In/Sys*
PG TPATASGLFT. . . .	pPVXSma	+/+
NW FDITNWLATPATASGLFT. . . .	pPVXSma-Z13	-/-
RG PGRAFVTIGTPATASGLFT. . . .	pPVXSma-p18	+/-
DR GPGRAFVTIDGTPATASGLFT. . . .	pPVXSma-p18DD	+/+
SA KQIASNENMETMKGTPATASGLFT. . . .	pPVXSma-NP	+/+
SAPASTTQATG STTTTKTAG ATPATASGLFT. . . .	pPVX201	+/+

*Presence (+) or absence (–) of symptoms on inoculated (In) and systemic (Sys) leaves.

Amino acids indicated in bold are those of the heterologous peptides; amino acids indicated in bold italic are nonconsensus residues of the wild-type, mutated or chimeric CP.

Nicotiana benthamiana plants were inoculated with vectors encoding the wild-type (pPVX201) (used as control) or the mutant (pPVXSma) virus, unmodified or encoding chimeric CPs (pPVXSma-Z13, pPVXSma-p18, pPVXSma-p18DD and pPVXSma-NP; see Fig. S1 in Supporting Information), and their stability was verified at both the genetic and protein levels, as described previously (Lico *et al.*, 2006, 2009; Fig. S2 in Supporting Information). The Z13 peptide fusion is known to interfere with both cell-to-cell and systemic movement, leading to low expression of the chimeric CP (Lico *et al.*, 2006). For this reason, to ensure the access of this viral genome to a larger number of cells, thus favouring expression and sampling for microscopy, experiments were also performed by inoculating *N. benthamiana* leaves with *Agrobacterium tumefaciens* GV3101 carrying the binary vector pGR106-Z13. This vector derives from pGR106 which encodes the entire PVX genome and induces the onset of viral infection (Lu *et al.*, 2003). In pGR106-Z13, the sequence encoding the wild-type CP was excised by *Sall-SpeI* digestion and substituted with the CP-Z13 fusion fragment obtained by the digestion of the vector pPVXSma-Z13 with the same restriction enzymes.

A first compelling discovery of our study was the existence of a direct correlation between the onset of infection symptoms on systemic leaves and the ability of the viral vectors to generate assembled virus particles (plants inoculated with pPVX201, pPVXSma, pPVXSma-p18DD or pPVXSma-NP). This correlation was verified by transmission electron microscopy (TEM) of 400-mesh grids precoated with a polyclonal antibody specific to CP (Adgen Phytodiagnostic, Ayr, UK) on which saps from inoculated and/or systemic infected leaves, 10 days post-inoculation (dpi), were adsorbed (immunosorbent electron microscopy, ISEM; Milne and Lesemann, 1984) (Fig. S3 in Supporting Information), and also by the examination of immunogold-labelled ultrathin sections of resin-embedded tissue fragments (Faoro *et al.*, 1991). No assembled virions were present in crude extracts or tissue fragments of leaves inoculated with constructs unable to produce systemic infection, i.e. leaf portions including chlorotic spots induced by inoculation with pPVXSma-p18 and asymptomatic leaves inoculated with pPVXSma-Z13 or agroinfiltrated with pGR106-Z13.

In particular, in leaves inoculated with pPVXSma-p18 (moving only cell to cell), in spite of the absence of assembled virions in

both cells and inside sieve elements (SEs), even at 10 dpi, the other typical traits of PVX infection, such as overproliferated endoplasmic reticulum (ER) and laminated inclusion components (LICs), were present (Bamunusinghe *et al.*, 2009) (Fig. 1A,B). At the same infection time, immunogold staining experiments also showed the presence of CP aggregated in compact solid masses of fibrous material surrounding LICs (Fig. 1B,C). CP accumulation in these masses, rather than its assembly into virions, could be ascribed to the intrinsic charge of the p18 peptide that could impair the ordered arrangement of CP units around viral RNA. This hypothesis was supported by the fact that CP fusion to a pI-modified version of this peptide (p18DD; Table 1) resulted in assembly and systemic movement. At 10 dpi in inoculated and systemic leaves of plants infected with pPVXSma-p18DD, LICs were surrounded by CP aggregates, but also by stacked assembled virus particles (Fig. 1D–G).

Z13 peptide fusion to CP has also been shown previously to be detrimental to viral movement (Lico *et al.*, 2006) but, in this case, even cell-to-cell transfer was impaired. Because of the absence of visible signs of infection on plant tissues inoculated with pPVXSma-Z13, the presence of damaged/infected cells was verified by light microscopy after staining with Evans blue at 4 and 10 dpi. Staining was carried out by boiling leaves in a mixture of phenol, lactic acid, glycerol and distilled water (1:1:1:1) containing 20 mg/L Evans blue, as described previously (Faoro and Iriti, 2005; Keogh *et al.*, 1980). These experiments revealed the presence in the upper leaf epidermis and in the palisade of a few scattered groups of three to four stained cells, probably corresponding to the cells in which the recombinant vector entered and was actively expressed (Fig. 1H). The number of these cells did not increase at 10 dpi, confirming the inability of the CP-Z13 fusion to contribute to movement (Fig. 1L,M). Evans staining and CP immunolocalization on leaves punctured with needles immersed in *A. tumefaciens* cultures (Ozeki *et al.*, 2009) carrying the binary vectors pGR106 and pGR106-Z13 further confirmed the results obtained with pPVXSma-Z13, and demonstrated the reliability of Evans blue staining in detecting infected cells (Fig. S4 in Supporting Information). In this case, the presence of a few stained cells can probably be ascribed to the expression of the recombinant vector in every single cell in which the plasmid was transported by *Agrobacte-*

Fig. 1 (A) Electron micrograph of a damaged cell close to a functional uninfected sieve element (SE) in a pPVXSma-p18-inoculated *Nicotiana benthamiana* leaf, containing overproliferated endoplasmic reticulum (ER) (arrow). (B) Coat protein immunogold labelling (CP-IGL) of a pPVXSma-p18-inoculated leaf, showing a vascular parenchyma cell with laminated inclusion components (LICs) surrounded by amorphous CP aggregates (enlarged in C). (D) CP-IGL of a pPVXSma-p18DD-inoculated leaf, showing the cytoplasm of a xylem parenchyma cell containing LICs, proteinaceous material and virions (enlarged in E). (F) CP-IGL of a PVXSma-p18DD infected systemic leaf, showing a mesophyll cell containing LICs surrounded by CP (arrow). (G) PVXSma-p18DD virions (arrows) in a mesophyll cell in a systemic infected leaf. (H) Light microscopy of an Evans blue-stained pPVXSma-Z13-inoculated leaf at 4 days post-inoculation (dpi). (I) Evans blue-stained leaf inoculated with phosphate-buffered saline as a control. (J) Portion of a leaf treated as in (H) at 10 dpi (enlargement in K). (L) Section of a pGR106-Z13 agroinfiltrated leaf showing LICs. (M) CP-IGL of a pGR106-Z13 infected cell with large amounts of CP accumulated in the cytoplasm among the ER endomembrane system (arrow). Bars: (A, D, F, L, M) 500 nm; (B) 1 μ m; (C, E, G) 250 nm; (J) 100 μ m; (H, I) 50 μ m; (K) 20 μ m.

with *A. tumefaciens* carrying pGR106-Z13 (following the protocol described in Buriani *et al.*, 2011). These experiments, by demonstrating the ability of this chimeric viral genome to induce the onset of infection, as indicated by the presence of LICs (Fig. 1N), showed CP accumulations mainly in proximity to ER cisternae, and possibly anchored to them (Fig. 1O). Assembled virus particles could not be detected in any of the examined cells.

All the presented data reinforce the concept that CP has pivotal and different functions in viral movement, and focus the attention on the role of its N-terminus in intra- and intermolecular interactions during PVX assembly. It has been demonstrated previously that this portion of the protein is rich in serine/threonine residues used as phosphorylation sites for unpackaging (Atabekov *et al.*, 2001), and that phosphorylation mediates the formation of a water shell that stabilizes virion structure (Baratova *et al.*, 2004). Here, we showed that the mutant PVXSma, with an N-terminally-deleted CP, forms rigid rod-shaped aggregates of intertwined particles, whereas PVXSma-NP, carrying a peptide that partially restores the N-terminus length and adequate serine/threonine content, is morphologically comparable with wild-type PVX (Fig. S3). Structural features similar to those of PVXSma have been described previously with a recombinant PVX mutant lacking amino acids 7–31 of CP (Chapman *et al.*, 1992) and with trypsin-treated virions (Tremaine and Agrawal, 1972). Thus, it is plausible that the absence in PVXSma of the N-terminus of CP is the reason why virions tend to aggregate. Experiments aimed to evaluate the infection progression efficiency of the different chimeric viruses into plants, by estimating the CP content as a percentage of total soluble proteins (TSPs) in leaf extracts, indicated that PVXSma is less virulent, possibly because of the absence of the serine/threonine phosphorylation sites that impair disassembly. The CP percentage in extracts obtained by homogenizing 100 mg of symptomatic

rium. Possible explanations for our microscopy observations are that the Z13 peptide, originally belonging to the transmembrane portion of gp41 involved in human immunodeficiency virus type 1 fusion to host cells (Salzwedel *et al.*, 1999; Zwick *et al.*, 2001), induces either the irreversible association of CP to cellular membranes or impairs CP interaction with TGBp1 for the formation of RNP (Atabekov *et al.*, 2007). The first of these hypotheses was confirmed by immunogold staining of tissues vacuum infiltrated

Fig. 2 (A) Evans blue-stained PVXSma-infected systemic leaf, showing infected cells around class IV and V (minor) veins (arrow). (B) Evans blue staining of a cluster of PVXSma-infected cells at the end of a class V vein (the arrow indicates the terminal part of a xylem element, visible by merging two images at different focus planes). (C) Evans blue staining of a cluster of PVXSma-infected cells at the end of a class V vein. (D) Coat protein immunogold labelling (CP-IGL) of a wild-type virus-infected systemic leaf, showing virus accumulation (V) in a vascular parenchyma cell (frame enlarged in E) (X, mature xylem; Ch, chloroplasts). (F) CP-IGL of a developing xylem element in a systemic leaf, showing wild-type virus accumulation in the residual cytoplasm (C) (M, mitochondria) (frame enlarged in G). (H) CP-IGL of a differentiated xylem element containing wild-type virus (frame enlarged in I). Bars: (A) 200 μ m; (B, C) 50 μ m; (D) 1 μ m; (E, F, H) 500 nm; (G, I) 100 nm.

systemic leaf tissues sampled at 10 dpi was determined through an enzyme-linked immunosorbent assay (ELISA) performed as described previously (Lico *et al.*, 2006). The results indicated that the wild-type virus was the most invasive, with CP representing about 6.5% of TSP, followed by PVXSma-NP (5% TSP), PVXSma-p18DD (1.5% TSP) and PVXSma (<0.5% TSP). Similar results were obtained by counting virus particles on 10 square grids randomly chosen in ISEM specimens. In this case, no significant differences were found between wild-type PVX and PVXSma-NP in terms of the number of particles, whereas PVXSma-p18DD particles were a fifth of wild-type particles (data not shown).

Fig. 3 (A) Coat protein immunogold labelling (CP-IGL) of the longitudinal section of a class V vein in a PVXSma-infected systemic leaf at 10 days post-inoculation (dpi) (X, mature xylem; XP, xylem parenchyma; SE, sieve element) (the cross-section of a similar vein is shown in inset B). (C) CP-IGL of the xylem framed in (A) showing PVXSma accumulation. (D) CP-IGL of the PVXSma-infected xylem parenchyma cell framed in (A) containing disordered masses of virus particles. (E) CP-IGL of the PVXSma-infected immature SE framed in (A). (F) Light microscopy of a longitudinal leaf section after CP-IGL and silver enhancement showing the CP (dark spots indicated by arrows) in the terminal part of class III veins and xylem elements (inset: negative control not labelled with anti-CP). (G) Section in (F) under polarized epifluorescence with CP evidenced as bright green spots (arrows) (inset: negative control under polarized epifluorescence). Bars: (A) 5 μ m; (B) 10 μ m; (C–E) 250 nm; (F, G insets) 20 μ m.

These data indicate the existence of a correlation between the amount of CP and the number of assembled virions.

The aggregation of PVXSma virions could also explain why, at 10 dpi, light microscopy observations of infected systemic leaves

stained with Evans blue revealed the presence of clusters of damaged/infected cells also at the extremities of class V veins, different from that observed in wild-type infected plants (Fig. 2A–C and Fig. S5 in Supporting Information). It has been suggested that protein and virus unloading in cells neighbouring veins of different classes is regulated by proteolysis (Mekuria *et al.*, 2008). In this view, compact PVXSma aggregates, different from isolated wild-type virions, could escape from proteasome-mediated degradation that normally blocks viral diffusion from minor veins.

The ultrastructural features of plant tissues infected with wild-type and PVXSma viruses also gave a new interesting insight into the strategy adopted by the virus to infect plants. TEM of immunogold-labelled leaves at 10 dpi with pPVX201 showed the presence of clear signs of infection in minor class veins, not only at the level of vascular parenchyma cells and phloem elements, but also into xylem elements either developing (still containing cytoplasm and organelles) or fully differentiated (Fig. 2D–I). Similarly, PVXSma virions were localized in differentiating and mature xylem elements not only by electron microscopy, but also by immunogold labelling of thick sections incubated with the anti-CP antibody, followed by silver enhancement (Helliot *et al.*, 2007) (Fig. 3A–G). To our knowledge, this is the first report of this particular PVX localization pattern, which indicates that, before programmed cell death is activated, the virus exploits these differentiating cells for replication like other plant viruses (Paliwal, 1970; Verchot *et al.*, 2001). Further experiments are necessary to assess whether PVX also uses these vessels to travel throughout the plant.

In conclusion, together, the results shown herein demonstrate definitively that virus particle assembly is dispensable for cell-to-cell movement, but essential for long-distance transfer, probably not only through the phloem, but also through the xylem. Moreover, these data reinforce the concept that the N-terminal portion of CP has a pivotal role in virus particle morphology and stability, and strongly influences the efficiency of spread of the infection.

ACKNOWLEDGEMENTS

This work was partially supported by a Grant from the Italian Foreign Affairs Ministry within the framework of the Bilateral Projects of Great Relevance. We acknowledge Professor David Baulcombe and Plant Bioscience Limited for supplying the pGR106 vector used in this study, and Professor Luca Santi and Dr Mario Tavazza for critical reading of the manuscript.

REFERENCES

- Atabekov, J., Dobrov, E., Karpova, O. and Radionova, N. (2007) Potato virus X: structure, disassembly and reconstitution. *Mol. Plant. Pathol.* **8**, 667–675.
- Atabekov, J.G., Rodionova, N.P., Karpova, O.V., Kozlovsky, S.V., Novikov, V.K. and Arkhipenko, M.V. (2001) Translational activation of encapsidated Potato virus X RNA by coat protein phosphorylation. *Virology*, **286**, 466–474.
- Bamunusinghe, D., Hemenway, C.L., Nelson, R.S., Sanderfoot, A.A., Ye, C.M., Silva, M.A.T., Payton, M. and Verchot-Lubicz, J. (2009) Analysis of potato virus X replicase and TGBp3 subcellular locations. *Virology*, **393**, 272–285.
- Baratova, L.A., Fedorova, N.V., Dobrov, E.N., Lukashina, E.V., Kharlanov, A.N., Nasonov, V.V., Serebryakova, M.V., Kozlovsky, S.V., Zayakina, O.V. and Rodionova, N.P. (2004) N-terminal segment of Potato virus X coat protein subunits is glycosylated and mediates formation of a bound water shell on the virion surface. *Eur. J. Biochem.* **271**, 3136–3145.
- Baulcombe, D.C., Chapman, S. and Santa Cruz, S. (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* **7**, 1045–1053.
- Buriani, G., Mancini, C., Benvenuto, E. and Baschieri, S. (2011) Plant heat shock protein 70 as carrier for immunization against a plant-expressed reporter antigen. *Transgenic Res.* **20**, 331–344.
- Chapman, S., Hills, G., Watts, J. and Baulcombe, D. (1992) Mutational analysis of the coat protein gene of Potato virus X: effects on virion morphology and viral pathogenicity. *Virology*, **191**, 223–230.
- Faoro, F. and Iriti, M. (2005) Cell death behind invisible symptoms: early diagnosis of ozone injury. *Biol. Plant.* **49**, 585–592.
- Faoro, F., Tornaghi, R. and Belli, G. (1991) Localization of Closteroviruses on grapevine thin sections and their identification by immunogold labelling. *J. Phytopathol.* **133**, 297–306.
- Helliot, B., Panis, B., Busogoro, J.P., Sobry, S., Poumay, Y., Raes, M., Swennen, R. and Lepoivre, P. (2007) Immunogold silver staining associated with epifluorescence for Cucumber mosaic virus localisation on semi-thin sections of banana tissues. *Eur. J. Histochem.* **51**, 153–158.
- Ju, H.J., Brown, J.E., Ye, C.M. and Verchot-Lubicz, J. (2007) Mutations in the central domain of Potato virus X TGBp2 eliminate granular vesicles and virus cell-to-cell trafficking. *J. Virol.* **81**, 1899–1911.
- Ju, H.J., Ye, C.M. and Verchot-Lubicz, J. (2008) Mutational analysis of PVX TGBp3 links subcellular accumulation and protein turnover. *Virology*, **375**, 103–117.
- Karpova, O.V., Zayakina, O.V., Arkhipenko, M.V., Sheval, E.V., Kiselyova, O.I., Poljakov, V.Y., Yaminsky, I.V., Rodionova, N.P. and Atabekov, J.G. (2006) Potato virus X RNA-mediated assembly of single-tailed ternary 'coat protein–RNA–movement protein' complexes. *J. Gen. Virol.* **87**, 2731–2740.
- Keogh, R.C., Deverall, B.J. and Mcleod, S. (1980) Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. *Trans Br. Mycol. Soc.* **74**, 329–333.
- Lico, C., Capuano, F., Renzone, G., Donini, M., Marusic, C., Scaloni, A., Benvenuto, E. and Baschieri, S. (2006) Peptide display on Potato virus X: molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles. *J. Gen. Virol.* **87**, 3103–3112.
- Lico, C., Mancini, C., Italiani, P., Betti, C., Boraschi, D., Benvenuto, E. and Baschieri, S. (2009) Plant-produced Potato virus X chimeric particles displaying an influenza virus-derived peptide activate specific CD8+ T cells in mice. *Vaccine*, **27**, 5069–5076.
- Lough, T.J., Netzler, N.E., Emerson, S.J., Sutherland, P., Carr, F., Beck, D.L., Lucas, W.J. and Forster, R.L. (2000) Cell-to-cell movement of Potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. *Mol. Plant–Microbe Interact.* **13**, 962–974.
- Lu, R., Malcuit, I., Moffet, P., Ruiz, M.T., Peart, J., Wu, A.J., Rathjen, J.P., Bendahmane, A., Day, L. and Baulcombe, D.C. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J.* **22**, 5690–5699.
- Lukashina, E., Badun, G., Fedorova, N., Ksenofontov, A., Nemykh, M., Serebryakova, M., Mukhamedzhanova, A., Karpova, O., Rodionova, N., Baratova, L. and Dobrov, E. (2009) Tritium planigraphy study of structural alterations in the coat protein of Potato virus X induced by binding of its triple gene block 1 protein to virions. *FEBS J.* **276**, 7006–7015.
- Mekuria, T., Bamunusinghe, D., Payton, M. and Verchot-Lubicz, J. (2008) Phloem unloading of Potato virus X movement proteins is regulated by virus and host factors. *Mol. Plant–Microbe Interact.* **21**, 1106–1117.
- Milne, R.G. and Lesemann, D.E. (1984) Immunosorbent electron microscopy in plant virus studies. *Methods Virol.* **8**, 85–101.
- Ozeki, J., Hashimoto, M., Komatsu, K., Maejima, K., Himeno, M., Senshu, H., Kawanishi, T., Kagiwada, S., Yamaji, Y. and Namba, S. (2009) The N-terminal region of the *Plantago asiatica* virus coat protein is required for cell-to-cell movement but is dispensable for virion assembly. *Mol. Plant–Microbe Interact.* **22**, 677–685.
- Paliwal, Y.C. (1970) Electron microscopy of Bromegrass mosaic virus in infected leaves. *J. Ultrastruct. Res.* **30**, 491–502.
- Salzwedel, K., West, J.T. and Hunter, E. (1999) A conserved tryptophan-rich motif in the membrane-proximal region of the Human immunodeficiency virus type 1 gp41

- ectodomain is important for Env-mediated fusion and virus infectivity. *J. Virol.* **73**, 2469–2480.
- Samuels, T.D., Ju, H.J., Ye, C.M., Motes, C.M., Blancaflor, E.B. and Verchot-Lubicz, J. (2007) Subcellular targeting and interactions among the Potato virus X TGB proteins. *Virology*, **367**, 375–389.
- Santa Cruz, S., Chapman, S., Roberts, A.G., Roberts, I.M., Prior, D.A.M. and Oparka, K.J. (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proc. Natl. Acad. Sci. USA*, **93**, 6286–6290.
- Santa Cruz, S., Roberts, A.G., Prior, D.A., Chapman, S. and Oparka, K.J. (1998) Cell-to-cell and phloem-mediated transport of Potato virus X. The role of virions. *Plant Cell*, **10**, 495–510.
- Schönknecht, G., Brown, J.E. and Verchot-Lubicz, J. (2008) Plasmodesmata transport of GFP alone or fused to potato virus X TGBp1 is diffusion driven. *Protoplasma*, **232**, 143–152.
- Tremaine, J.H. and Agrawal, H.O. (1972) Limited proteolysis of Potato virus X by trypsin and plant proteases. *Virology*, **49**, 735–744.
- Verchot, J., Driskel, B.A., Zhu, Y., Hunger, R.M. and Littlefield, L.J. (2001) Evidence that Soilborne wheat mosaic virus moves long distance through the xylem in wheat. *Protoplasma*, **218**, 57–66.
- Verchot-Lubicz, J., Ye, C.M. and Bamunusinghe, D. (2010) Varied movement strategies employed by triple gene block-encoding viruses. *Mol. Plant–Microbe Interact.* **23**, 1231–1247.
- Zayakina, O., Arkhipenko, M., Kozlovsky, S., Nikitin, N., Smirnov, A., Susi, P., Rodionova, N., Karpova, O. and Atabekov, J. (2008) Mutagenic analysis of Potato virus X movement protein (TGBp1) and the coat protein (CP): in vitro TGBp1–CP binding and viral RNA translation activation. *Mol. Plant Pathol.* **9**, 37–44.
- Zwick, M.B., Labrijn, A.F., Wang, M., Spenlehauer, C., Saphire, E.O., Binley, J.M., Moore, J.P., Stiegler, G., Katinger, H., Burton, D.R. and Parren, P.W. (2001) Broadly neutralizing antibodies targeted to membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J. Virol.* **75**, 10 892–10 905.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Schematic representation of viral expression vectors and *cp* gene regions. (A) pPVX201. Grey square, multicloning site; 35S, 35S promoter of cauliflower mosaic virus (CaMV); black square, *Agrobacterium tumefaciens nopaline synthase* gene transcription terminator; RdRP, RNA-dependent RNA polymerase; M, triple gene block encoding TGBp1, TGBp2 and TGBp3 proteins; CP, coat protein. Black arrows, *cp* gene original and duplicated subgenomic promoters. (B–D) pPVX201, pPVXSma and chimeric *cp* gene regions (horizontally striped box, sequence encoding the heterologous peptide). Major restriction sites are reported.

Fig. S2. Analysis of wild-type, mutated and chimeric coat proteins (CPs). Separation by 13.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified viruses or protein leaf extracts followed by silver staining (top panel) or Western blotting (by membrane hybridization with the

anti-Potato virus X (PVX) CP polyclonal antibody) (bottom panel). Lane 1, ColorBurst molecular weight marker (Sigma Chemical Co., St. Louis, MO, USA); lane 2, 300 ng purified wild-type PVX; lane 3, 200 ng purified PVXSma; lane 4, 100 ng purified PVXSma-NP; lane 5, 200 ng purified PVXSma-p18DD; lane 6, 1 µg protein extract from a pPVXSma-p18-inoculated leaf; lane 7, 1 µg protein extract from a pGR106-Z13 agroinfiltrated leaf; lane 8, 1 µg protein extract from an untreated leaf.

Fig. S3. Morphological analysis of wild-type, mutated and chimeric Potato virus X (PVX) by transmission electron microscopy (TEM). (A) Wild-type PVX. (B) PVXSma. (C) PVXSma-p18DD. (D) PVXSma-NP. Bars in micrographs, 250 nm; bars in insets, 25 nm.

Fig. S4. Comparison of virus spread from the inoculation site (asterisk), 5 days post-inoculation (dpi), by Evans blue staining (A–C) and immunogold labelling (followed by silver enhancement; Helliot *et al.*, 2007) carried out on thick tangential sections throughout the inoculation sites (D–G); the two techniques were used on half of the same leaf punctured with thin needles immersed in *Agrobacterium tumefaciens* cultures carrying different binary vectors. (A) Leaf inoculated with the binary vector pGR106 [wild-type Potato virus X (PVX)], showing numerous damaged cells (stained in blue) caused by virus spread around the inoculation site. (B) Leaf inoculated with the binary vector pGR106-Z13, showing damaged cells only at the inoculation site, indicating that no virus spread occurred. (C) Evans blue staining of a leaf inoculated with the binary vector pBI121, as control, showing that stained cells are only those damaged by the needle. (D) Thick section through an inoculation site similar to that framed in (A), showing a comparable pattern of virus spread, highlighted by silver–yellow brilliant fluorescence (arrows) caused by coat protein (CP) immunolabelling. (E) Thick section through an inoculation site similar to that framed in (B), showing that the Z13 mutant, highlighted by silver–yellow brilliant fluorescence (arrows) caused by CP immunolabelling, is only localized in a few cells at the inoculation site (arrows). (F, G) Immunolabelling with pre-immune serum, as negative controls, of serial sections of specimens in (D) and (E), respectively. Bars: (A–C) 100 µm; (D–G) 20 µm.

Fig. S5. (A) Evans blue staining of a wild-type Potato virus X (PVX)-infected systemic leaf at 10 days post-inoculation (dpi), showing infected cells only along class III veins. (B) Evans blue staining of a leaf inoculated with phosphate-buffered saline. Bars: (A) 100 µm; (B) 200 µm.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.