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Complementation of a potato virus X mutant mediated by bombardment of plant tissues with cloned viral movement protein genes

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Microprojectile bombardment was used to examine the transport function of the 25 kDa movement protein (MP) encoded in the triple gene block of potato virus X (PVX). A 25 kDa MP-defective full-length cloned PVX genome carrying a *β*-glucuronidase (GUS) reporter gene was co-bombarded with 35S promoter constructs containing either the 25 kDa MP gene of wild-type PVX, the MP gene of either of two tobamoviruses (tomato mosaic virus or crucifer tobamovirus), red clover necrotic mosaic dianthovirus (RCNMV) or brome mosaic bromovirus (BMV). When inoculated alone, the MP-defective PVX was unable to move out of the inoculated cell, as visualized by *in situ* staining for GUS activity. However, cell-to-cell movement of the mutant PVX genome was restored by co-inoculation with 35S constructs containing the MP cDNA of PVX, either tobamovirus or RCNMV. The BMV MP construct did not complement movement of the defective PVX. These results show that co-bombardment of cDNA of an MP-defective virus with plasmids designed to express MP of other viruses could be used as a fast and simple method for *trans*-complementation experiments.

Introduction

Unlike tobacco mosaic virus (TMV) and many other viruses having a single movement protein (MP) gene, the genomes of potexviruses, carlaviruses, hordeiviruses and some furoviruses encode three MPs in the overlapping open reading frames (ORFs) of a triple gene block (TGB) (Morozov *et al*., 1989; Rupasov *et al*., 1989; Koenig *et al*., 1996). Mutations in each of the TGB ORFs disable the virus transport function (Petty & Jackson, 1990; Beck *et al*., 1991; Gilmer *et al*., 1992; Angell *et al*., 1996). The 5′-most ORF in the TGB is the largest and codes for a protein [TGBp1, a 25 kDa MP in potato virus X (PVX)] possessing ATPase and RNA-binding activities (Skryabin *et al*., 1988; Rouleau *et al*., 1994; Kalinina *et al*., 1996; Bleykasten *et al*., 1996; Donald *et al*., 1995, 1997). Two smaller TGB-encoded proteins are most likely associated with membranes and cell walls (Morozov *et al*., 1989; Richards & Tamada, 1992; Donald *et al*., 1995; Hefferon *et al*., 1997). In addition to the TGB proteins, PVX coat protein is also required for cell-to-cell movement (Chapman *et al*., 1992*a*).

Different experimental approaches have shown that a defect in a viral MP can be complemented by the MP of a second, unrelated helper virus. The original demonstration that MPs of different viruses are functionally interchangeable involved double infections with movement-defective isolates and helper viruses (for references, see Atabekov & Taliansky, 1990). In a modification of this approach, it was shown that MP produced in transgenic plants could complement a transport-deficient virus (Deom *et al*., 1987; Ziegler-Graff *et al*., 1991; Kaplan *et al*., 1995). In a third type of complementation experiment, a recombinant virus genome was constructed with the wild-type MP gene replaced by the MP gene from a heterologous virus (Ziegler-Graff *et al*., 1991; Nejidat *et al*., 1991; De Jong & Ahlquist, 1992; Giesman-Cookmeyer *et al*., 1995; Solovyev *et al*., 1996, 1997). The ability of the inserted MP gene to facilitate cell-to-cell movement of the hybrid virus provided insights into the functional compatibility of the MPs of different viruses.

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Table 1. List of plant viral MP genes used for bombardment-mediated transient complementation of PVX movement

MP gene (plasmid name)	Plasmid vector	Source of gene	Coordinates of cloned gene in plasmid viral RNA genome*
PVX 25K (pRT-PVX.25K)	pRT101	PCR product	4485–5250[a]
PVX TGB (pRT-PVX.TGB)	pRT103	PCR product	4485–5711[a]
ToMV 30K (pRT-ToMV.30K)	pRT103	PCR product	4905–5760[b]
Cr-TMV 30K (pRT-CrTMV.30K)	pRT101	Yu. L. Dorokhov	4648–6312[c] (+CP)
RCNMV 35K (pRT-RCNMV.35K)	pRT103	S. A. Lommel	(RNA2-) 80–1033[d]
BMV 32K (pRT-BMV.32K)	pRT103	PCR product	(RNA3-) 92–1003[e]

* References: *a*, Skryabin *et al.* (1988); *b*, Ohno *et al.* (1984); *c*, Dorokhov *et al.* (1994); *d*, Lommel *et al.* (1988); *e*, Ahlquist *et al.* (1981).

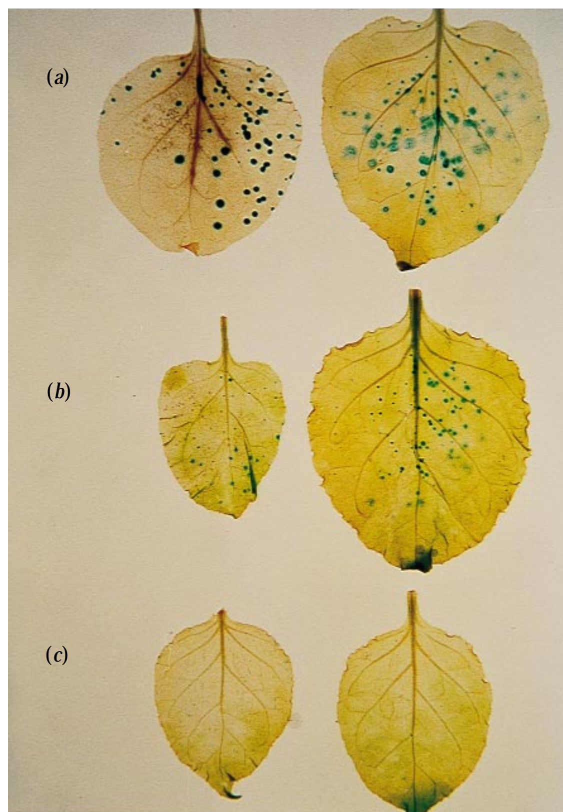

Fig. 1

Fig. 1. Histochemical analysis of GUS activity in pairs of young and old leaves of *N. benthamiana* bombarded with: (*a*) parental pPVX.GUS; (*b*) a mixture of pPVX.GUS-Bsp and pRT-PVX.TGB; or (*c*) pPVX.GUS-Bsp. Infection foci were photographed 2 days p.i. The presence of GUS activity is indicated by the blue staining.

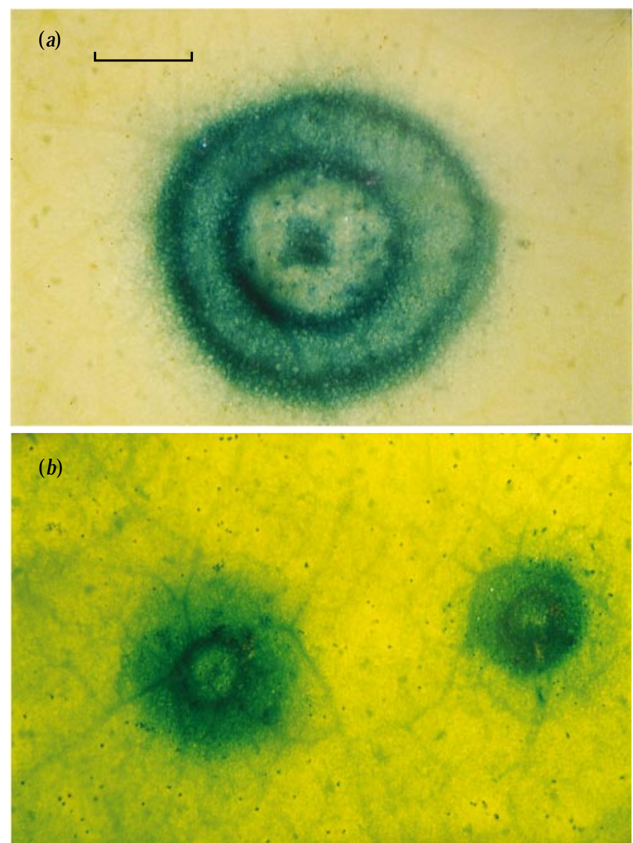

Fig. 3

Fig. 3. Histochemical analysis of GUS activity in leaves of flowering *N. benthamiana* plants bombarded with (*a*) parental pPVX.GUS or (*b*) a mixture of pPVX.GUS-Bsp and pRT-PVX.TGB. Infection foci were photographed at 3 days p.i. to show concentric ring effect. The bar in (*a*) represents 400 µm.

Here, we describe a new experimental approach for complementation studies based on microprojectile bombardment (Sanford, 1988), which simultaneously introduces a full-length genomic cDNA of a movement-defective virus and a plasmid containing the heterologous MP gene. Microprojectile bombardment has already been applied successfully for

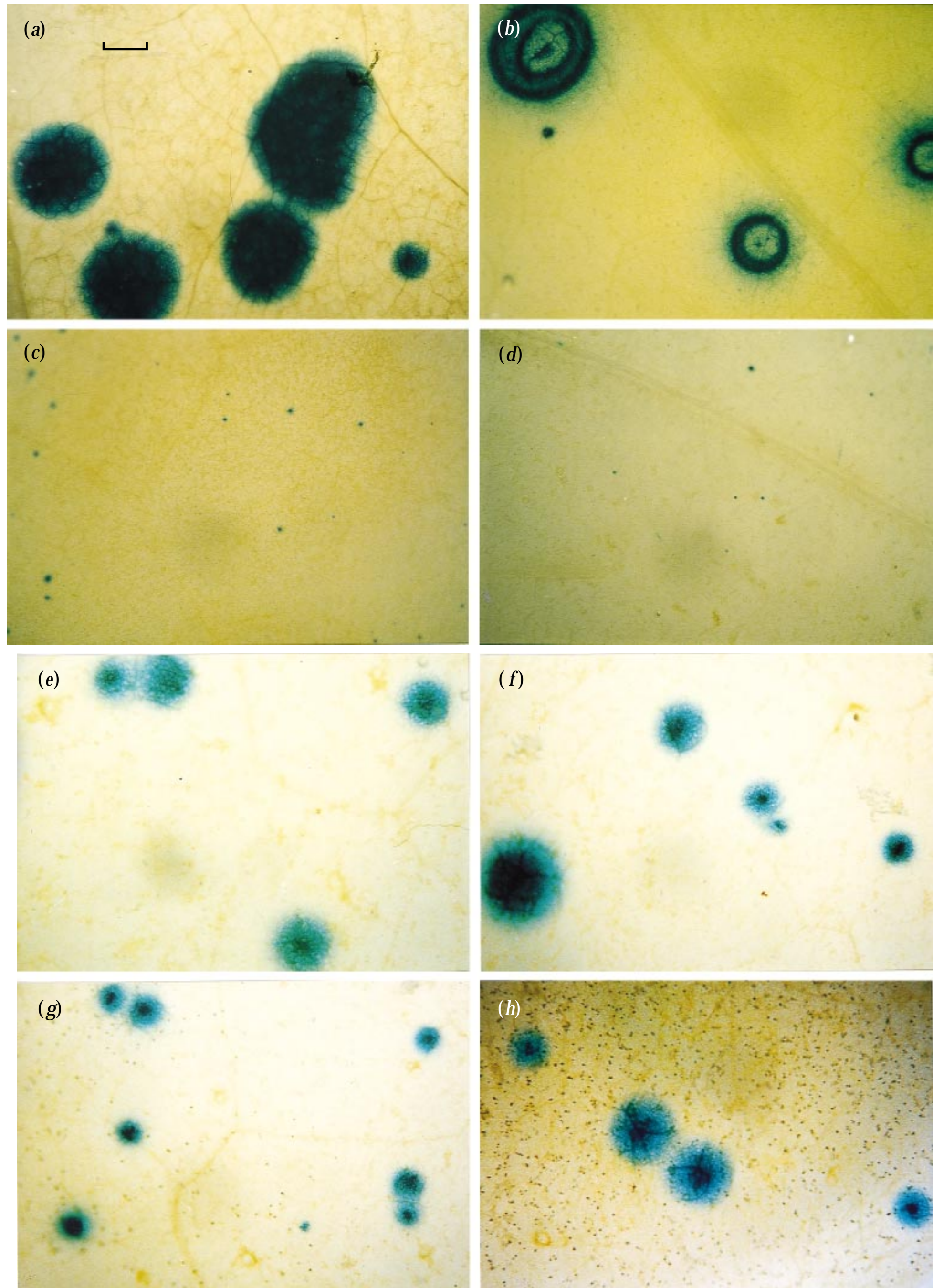

Fig. 2. Histochemical analysis of GUS activity in leaves of *N. benthamiana* bombarded with: (*a*) and (*b*) parental pPVX.GUS; (*c*) pFF19G; (*d*) pPVX.GUS-Bsp; or mixtures of pPVX.GUS-Bsp with pRT-PVX.25K (*e*), pRT-ToMV.30K (*f*), pRT-CrTMV.30K (*g*) or pRT-RCNMV.35K (*h*). Infection foci were photographed at 2 days p.i. The bar in (*a*) represents 400 μm. Statistics of foci for the examples presented are: pPVX.GUS [mean = 917·7 μm, SD = 367·8, standard error (SE) = 67·1]; pPVX.GUS-Bsp (mean = 30·4 μm, SD = 9·7, SE = 1·9); pRT-PVX.25K (mean = 454·0 μm, SD = 136·9, SE = 30·6); pRT-ToMV.30K (mean = 460·0 μm, SD = 161·8, SE = 36·2); pRT-CrTMV.30K (mean = 303·0 μm, SD = 76·0, SE = 17·1); and pRT-RCNMV.35K (mean = 441·0 μm, SD = 185·2, SE = 41·4).

transient gene expression in plant tissues (Godon *et al.*, 1993) and for plant infection with the cloned genomes of DNA- or RNA-containing viruses (Hagen *et al.*, 1994; Gal-On *et al.*, 1995). We report that transient complementation of a movement-defective *β*-glucuronidase (GUS)-tagged PVX can be readily detected by fast and simple experiments using co-bombardment of viral cDNA with 35S promoter-based plasmids designed to express MP gene(s).

Methods

■ All recombinant DNA procedures were carried out by standard methods (Sambrook *et al.*, 1989) in *Escherichia coli* strains DH5α and XL-1B. To clone PCR-amplified MP genes encoded by PVX (TGBp1 and the complete TGB), tomato mosaic tobamovirus (ToMV), red clover necrotic mosaic dianthovirus (RCNMV) and brome mosaic bromovirus (BMV), we used the 35S promoter-based pRT plasmids (Töpfer *et al.*, 1987) (Table 1). Plasmids were designated pRT-PVX.25K, pRT-PVX.TGB, pRT-ToMV.30K, pRT-RCNMV.35K and pRT-BMV.32K, respectively. Cloned RCNMV RNA-2 cDNA containing the MP gene was kindly provided by S. A. Lommel. The plasmid pRT-CrTMV.30K, carrying the MP and coat protein genes of crucifer tobamovirus (Cr-TMV) under 35S promoter control, was kindly provided by Yu. L. Dorokhov. To construct the full-length PVX genome with a mutated 25 kDa protein gene (pPVX.GUS-Bsp), plasmid pPVX.GUS, containing GUS-gene-tagged PVX genome under the control of the 35S promoter, was used. This tagged PVX cDNA was analogous to that in pGC3 (Chapman *et al.*, 1992*b*). pPVX.GUS was linearized by *Bsp*120I (position 4945 in the PVX genome), blunt-ended with Klenow fragment and religated, to give pPVX.GUS-Bsp. In this report, progeny viruses with a GUS reporter gene are referred to as PVX.GUS and PVX.GUS-Bsp, respectively.

Particle bombardment was performed using the flying disk method (Daniell, 1993) with the high-pressure helium-based apparatus PDS-1000 (Bio-Rad). Briefly, for each series of shots, DNA was precipitated on tungsten particles with calcium chloride and ethanol after the addition, while vortexing, of 10 µl plasmid DNA (at 0·5–1·5 mg/ml) to 6 mg tungsten particles suspended in 100 µl 50% glycerol. The DNA-coated tungsten particles were kept in suspension in cold absolute ethanol (90 mg/ml). After sonication, 5 µl of this mixture was placed immediately on each plastic flying disk and used for bombardment when the particles had dried. A detached leaf of *Nicotiana benthamiana* (15–30 mm size) was placed in the centre of a plastic Petri dish and bombarded on a solid support at a target distance of 7 cm. Bombardment was done with a pulse of 1350 kPa helium gas in a vacuum chamber.

Inoculated leaves were sampled 24–72 h after bombardment. Replication and movement of PVX.GUS were monitored by histochemical detection of GUS expression as described (Jefferson, 1987). Samples were infiltrated with the colorimetric GUS substrate 5-bromo-4-chloro-3-indolyl *β*-D-glucuronide (X-Gluc) at 600 µg/ml, modified (De Block & Debrouwer, 1992) to limit the diffusion of the intermediate products of the reaction in 0·115 M phosphate buffer, pH 7·0, 3 mM potassium ferricyanide, 10 mM EDTA. After incubation overnight at 37 °C, leaves were fixed in 70% ethanol and examined by light microscopy.

Results and Discussion

A 25 kDa-MP-defective PVX was generated from a cDNA clone (Chapman *et al.*, 1992 *b*) of PVX.GUS placed under the control of the cauliflower mosaic virus 35S promoter. This mutant construct contained a four nucleotide insert after codon 154 in the first ORF of the TGB (pPVX.GUS-Bsp). As a result of this frameshift mutation, the 25 kDa PVX-encoded protein lacked 72 C-terminal amino acid residues including two motifs of the ATPase domain (Gorbalenya & Koonin, 1989; Morozov *et al.*, 1989).

Plasmids pPVX.GUS and pPVX.GUS-Bsp were used directly to bombard *Nicotiana benthamiana* leaves and movement of the virus was monitored by histochemical analysis of GUS (Fig. 1). With pPVX.GUS, the blue foci representing GUS expression developed 1–2 days p.i. (Figs 1*a* and 2*a*, *b*). These foci were similar to or slightly smaller in diameter than those produced after mechanical inoculation of *N. clevelandii* with PVX.GUS (Chapman *et al.*, 1992*b*; Angell & Baulcombe, 1995). In contrast, the blue foci on pPVX.GUS-Bsp-bombarded leaves were difficult to see with the naked eye (Fig. 1*c*). The infection foci were confined either to individual cells or to small groups of cells (Fig. 2*d*). The same pattern of GUS staining was produced by the 35S-GUS plasmid pFF19G (Timmermann *et al.*, 1990) containing only the GUS gene (Fig. 2*c*). These observations indicate that PVX.GUS-Bsp was not able to move out of the primarily inoculated cell. Occasional GUS staining in groups of adjacent cells is likely due either to diffusion of the chromogenic dye or simultaneous infection of a group of epidermal or mesophyll cells. As the mutation in pPVX.GUS-Bsp would not have affected virus replication (Angell *et al.*, 1996), we conclude that this PVX mutant was defective in cell-to-cell movement.

To test for transient complementation of the movement defect, pPVX.GUS-Bsp was co-bombarded with pRT-PVX.TGB which contains PVX TGB in a 35S promoter expression cassette. Staining for GUS at 2 days p.i. revealed blue foci, clearly visible to the naked eye (Fig. 1*b*). Similarly, cell-to-cell transport of the mutant PVX genome was restored by the presence of pRT-PVX.25K in initially infected cells (Fig. 2*e*). However, although the cell-to-cell spread of PVX.GUS-Bsp had been complemented, the blue infection foci were only 40–60% the size of those obtained with PVX.GUS (Figs 2*a*, *e* and 3*a*, *b*). Importantly, there was no increase in size of the blue spots beyond 2 days p.i. with pPVX.GUS-Bsp plus pRT-PVX.TGB or pRT-PVX.25K. In contrast, the regions of GUS staining in PVX.GUS-infected leaves continued to increase for 3–5 days p.i. and even spread into the vascular system (data not shown). It is logical to propose that complementation of movement-deficient PVX.GUS-Bsp was due to transient expression of the functionally active 25 kDa MP gene from pRT-PVX.TGB or pRT-PVX.25K constructs in cells doubly-inoculated upon co-bombardment.

Between 30% and 60% of blue foci in leaves bombarded with pPVX.GUS exhibited a concentric ring pattern of GUS staining around the primary bombarded cell (Figs 2*b* and 3*a*). This pattern was also produced in some of the complementation experiments, although the rings were smaller and more diffuse than those produced by pPVX.GUS (Fig. 3*b*). Such concentric rings of GUS staining were never observed after bombardment with pFF19G or pPVX.GUS-Bsp. We suggest that these rings, also reported by Heinlein *et al.* (1995) in *N. benthamiana* leaves infected with green-fluorescent-protein-tagged TMV, may reflect a diurnal variation in viral gene expression.

We have shown recently that movement of chimaeric variants of the TGB-containing barley stripe mosaic virus (BSMV) can be potentiated by artificially inserted MP genes of TMV and RCNMV (Solovyev *et al.*, 1996, 1997). To test the

specificity of PVX.GUS-Bsp complementation in the transient bombardment assay, we inoculated pPVX.GUS-Bsp with 35S-based plasmids containing the MP genes of tobamoviruses (ToMV or Cr-TMV), a dianthovirus (RCNMV) or a bromovirus (BMV) (Table 1). Cell-to-cell movement of PVX.GUS-Bsp was restored by co-bombardment with 35S constructs containing the MP cDNA of either tobamovirus or RCNMV (Fig. 2*f*, *g*, *h*). However, cell-to-cell movement of PVX.GUS-Bsp was not restored by co-bombardment with the plasmid pRT-BMV.32K (data not shown). These data agree with the observation that movement of chimaeric BSMV was not restored by inserting the BMV MP gene (A. G. Solovyev, E. I. Savenkov, V. Z. Grdzelishvili, S. Yu. Morozov & J. G. Atabekov, unpublished results), but are not easily reconciled with double-infection experiments in which defective cell-to-cell movement of PVX in a PVX-resistant plant (*Hordeum vulgare*) could be complemented by co-infection with BMV (Malyshenko *et al*., 1989). Perhaps the inability of the BMV MP to complement movement of defective PVX in the transient bombardment assay could be due to the fact that cell-to-cell movement of BMV itself requires the presence of BMV coat protein (Schmitz & Rao, 1996), whereas cell-to-cell movement of tobamoviruses and RCNMV are coat-protein-independent.

These data demonstrate that *trans*-complementation of a mutation in the TGBp1 gene of PVX by homologous or heterologous MPs can be examined readily by the transient bombardment assay. They also confirm observations made by ourselves and other groups (reviewed by Atabekov & Taliansky, 1990) that, in many cases, MP functions of unrelated viruses are compatible. This compatibility is apparently not affected by the monopartite or multipartite nature of the various virus MP systems tested.

Furthermore, these data also show, from the size and pattern of GUS staining, that the movement-deficient mutant of PVX had spread into many cells after co-bombardment with the MP constructs. Thus, the various MP genes expressed in a single cell can complement movement of the defective virus through several cell boundaries. These data agree with results from microinjection studies, which showed that TMV and RCNMV MPs are able to move from the initially injected cell (for references, see Mezitt & Lucas, 1996), and with the suggestion that the 25 kDa TGBp1 has the potential to move between cells, as proposed by Angell *et al*. (1996). Alternatively, the TGBp1 mRNA that is transiently expressed in the initially 'co-infected cell' could move from cell to cell, like viral RNA under the influence of the MPs, and synthesize new MP molecules in each secondary 'infected cell'. In either case, there would be a decreasing concentration gradient of the TGBp1 mRNA molecules and, as observed, spread of the defective virus would be transient and less extensive than with wild-type virus.

Microinjection has been used in numerous studies on virus cell-to-cell movement. Microinjection of virus particles (or purified MPs) together with FITC-labelled dextrans into plant leaf cells (Derrick *et al*., 1992) showed that MPs allow extensive and rapid trafficking of fluorescent dye and viral RNA across many cellular boundaries. An advantage of microinjection is that single cells can be targeted for analysis. This system has provided direct evidence for the cell-to-cell trafficking activity of several viral MPs and allowed mapping of their functional domains (for references, see Mezitt & Lucas, 1996).

However, the microinjection procedure is difficult to carry out on a large scale and it may perturb the plasmodesmal connections between cells. We would stress that transient complementation of tagged virus genomes, mediated by microprojectile bombardment, permits quick and quantitative analysis of virus movement. Moreover, using isolated, cloned MP genes will facilitate site-directed mutagenesis to analyse structure–function relationships in these proteins.

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This publication is dedicated by Joachim Schiemann to Professor Dr Benno Parthier, his esteemed academic teacher and supervisor of his thesis in Halle in the mid-1970s. Professor Parthier, Director of the Institute for Plant Biochemistry, Halle, and President of the 'Deutsche Akademie der Naturforscher Leopoldina', celebrates his 65th anniversary on 21 August 1997.

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