Mutations in the coat protein gene of *Plum pox virus* suppress particle assembly, heterologous encapsidation and complementation in transgenic plants of *Nicotiana benthamiana*

Mark Varrelmann and Edgar Maiss

Institute of Plant Diseases and Plant Protection, University of Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany

Two different motifs in the coat protein (CP) of *Plum pox virus* (PPV) (R3015Q3016, D3059) were mutated by replacing the respective amino acids with others possessing different chemical properties. The mutated CP genes were introduced into an infectious full-length clone of PPV (p35PPV-NAT) to investigate their influence on systemic infection of transgenic wild-type PPV CP-expressing and non-transgenic plants of *Nicotiana benthamiana*. All mutants failed to establish systemic infections in non-transgenic *N. benthamiana* plants, but were complemented by intact CP in transgenic plants. Moreover, the CP-RQ-D mutant (carrying mutations in both the RQ and D motifs) was introduced into p35PPV-NAT engineered to express β-glucuronidase (GUS) for direct observation of systemic movement and particle assembly in *N. benthamiana* leaves. GUS-staining revealed that the CP mutant (RQ-D) was restricted to initially infected cells without forming virions. Systemic movement and particle assembly were restored in CP-transgenic *N. benthamiana* plants. Finally, transgenic *N. benthamiana* plants were generated that expressed each of the three mutated CP genes. Homozygous T2 lines were selected and tested for resistance to PPV. Immunogold labelling and electron microscopy revealed that heterologous encapsidation with challenging *Chilli veinal mottle virus* and *Potato virus Y* was suppressed in these lines. In addition, assembly mutants did not complement CP-defective p35PPV-NAT. The possible use of modified viral CP genes for the production of virus-resistant transgenic plants, thereby reducing the putative risks of heterologous encapsidation and complementation, is discussed.

Introduction

Since 1986, plant virus coat protein (CP) genes have been used widely to produce pathogen-derived resistance in various plant species. Today, this strategy is of increasing agronomic importance because resistance can be introduced more easily and more quickly compared with conventional breeding techniques.

It was initially anticipated that resistance operated through expression of the viral protein (Powell *et al.*, 1990; Lapidot *et al.*, 1993). Meanwhile, several studies concerning the mechanism of resistance demonstrated that, in most cases, resistance is RNA-mediated and is caused by co-suppression, post-transcriptional or homology-dependent gene silencing (for reviews see van den Boogaart *et al.*, 1998; Wasseneger & Pélissier, 1998). Lindbo & Dougherty (1992a) were the first to show that untranslatable potyviral genes are also able to confer resistance in transgenic plants. This has been confirmed for many host/virus combinations (for a review see Prins & Goldbach, 1996). In addition, Pang *et al.* (1997), Silva-Rosales *et al.* (1994) and Jacquet *et al.* (1998b) were able to produce virus resistance in transgenic plants by using shortened viral genes. Moreover, the authors could show that the viral genes were not necessary in their entirety for the resistance mechanism to be triggered.

The three CP-transgenic plants that have been approved for commercial release in the USA have, however, been transformed with full-length and translatable viral CP genes (White, 1999). Moreover, recent studies have shown that molecular interactions with challenging viruses in transgenic plants can lead to heterologous encapsidation, complementation and recombination (Balázs & Tepfer, 1997). These findings have raised concerns about potential biological and
environmental risks associated with virus-resistant transgenic plants.

In nature, heterologous encapsidation occurs in mixed infections between two related viruses (Rochow, 1977; Wen & Lister, 1991). In transgenic plants, with functional viral CPs expressed in every cell, CP subunits can be used by closely related viruses for heterologous encapsidation, as shown by Farinelli et al. (1992) for Potato virus Y (PVY) and by Maiss et al. (1995) for various potyviruses. Hence, the transgenic CP can transfer functions like vector specificity (Lecoq et al., 1993, 1994). To avoid the transfer of aphid transmission functions via heterologous encapsidation within the potyviruses, motifs involved in aphid transmission can be altered or removed.

Atreya et al. (1991) characterized the amino acid triplet DAG at the N terminus of the CP responsible for aphid transmission. Mutations in this motif, or the use of CPs of non-aphid-transmissible virus isolates, can prevent transmission. On the other hand, functions of the CP such as host specificity (Shukla et al., 1991) have been characterized poorly to date and cannot easily be removed from the CP gene.

No matter which function could be transferred via heterologous encapsidation to challenging viruses, it would be useful to prevent this phenomenon in transgenic plants. One possibility is to render the viral CP gene untranslatable by introducing stop codons via mutagenesis in all three possible open reading frames. Another possibility is to abolish the ability of the transgenic CP to form virus particles. This can be achieved by mutating the amino acid motifs RQ and D within the CP, conserved within the genus Potyvirus and assumed to form a salt bridge between α-helices (Dolja et al., 1991). The RQ and D motifs in the CP of Johnsongrass mosaic virus and PPV have been shown to be involved in particle assembly in Escherichia coli (Jagadish et al., 1991, 1993; Jacquet et al., 1998a). Dolja et al. (1994) mutated the RQ and D motifs in the CP of Tobacco etch virus (TEV) and introduced the mutated gene into TEV-gus (carrying the β-glucuronidase gene, gus) to investigate the participation of potyvirus CP in cell-to-cell and long-distance movement. The authors did not detect virus particles in protoplasts infected with RQ and D mutants of TEV-gus, even if the intact CP was supplied in trans. Rojas et al. (1997) demonstrated the contribution of the CP to cell-to-cell movement of potyviruses in microinjection experiments.

Complementation occurs in transgenic plants if a virus mutant, defective in one gene, is complemented in trans by the corresponding intact transgenically expressed protein. Osbourn et al. (1990) were able to complement a CP-defective Tobacco mosaic virus (TMV) and Holt & Beachy (1991) complemented a movement protein-defective TMV in transgenic plants expressing the respective intact viral protein. Kaplan et al. (1995) found similar complementation of a movement-defective Cucumber mosaic virus in transgenic plants expressing the intact 3a movement protein. In addition, Dolja et al. (1994, 1995) were able to complement CP-defective TEV. Jakab et al. (1997) detected complementation of a PVY CP frame-shift mutant with a functional CP supplied in trans and suggested that the use of virus-resistant transgenic plants synthesizing a functional viral protein might create new environmental niches for mutated viruses and could free the viral gene from natural selection pressure.

In the present study, experiments were carried out to detect suppression of particle assembly after mutation of the R$^{2011}$Q$^{2016}$ and D$^{2018}$ motifs within the CP of PPV. To verify particle assembly, replication and short-distance movement of PPV mutants in planta, a gus-tagged full-length clone of PPV (p35PPV-NAT) was used to detect virus particles from single infected cells by immunosorbent electron microscopy (ISEM). In addition, it must be demonstrated that heterologous encapsidation and complementation can be abolished in transgenic Nicotiana benthamiana plants expressing dysfunctional PPV CP, e.g. assembly-defective CPs. The results are discussed in the context of recommendations for the future generation of virus-resistant transgenic plants.

**Methods**

*Modification of the PPV-NAT CP gene for plant transformation and gene replacement in p35PPV-NAT.* The CP gene of p35PPV-NAT was cloned into a plant expression vector under the control of an enhanced 35S promoter (2×35S) from Cauliflower mosaic virus (CaMV) and the PPV 5′ nontranslated region (ntr) followed by the CaMV polyadenylation signal (pe35SL-NAT-CP). Mutagenesis was carried out by PCR, producing three different mutations in the CP; CP-RQ, CP-D and CP-RQ-D. Amino acids in the assembly motifs were replaced with others possessing different chemical properties (Fig. 1A). In the RQ motif, Arg$^{2015}$ (numbers indicate amino acid positions in PPV-NAT according to Maiss et al., 1992) was replaced by Asp and Gin$^{2016}$ by Val. In the D motif, Asp$^{2018}$ replaced by Lys and Phe$^{2019}$ was replaced by Ile. Simultaneously, recognition sequences for restriction endonucleases were introduced to allow verification of mutagenesis. Mutagenesis was confirmed by restriction enzyme digestion and additional sequencing of each CP gene.

For production of assembly-defective PPV-NAT, mutated CP genes were inserted directly into p35PPV-NAT leading to p35PPV-NAT-CP-RQ, -D and -RQ-D. To confirm that no additional mutations had been introduced into the full-length clone during the cloning and replication process in E. coli, wild-type CP was cloned back into the different mutated p35PPV-NAT. The latter were subsequently tested for infectivity on N. benthamiana plants.

*Construction of p35PPV-NAT-gus-CP and p35PPV-NAT-gus-CP-RQ-D.* The objective was to express a recombinant β-glucuronidase (gus) gene, introduced into the PPV-NAT genome with minimal protein fusions at the termini of the marker protein, and to use the viral Nla protease for processing. The junction of NIB and CP was selected for the insertion of the reporter gene; both viral proteins should remain unaffected. Briefly, the gus gene was amplified by PCR from pBl121 (Clontech), introducing recognition sequences for Ncol at the 5′ end and Xhol at the 3′ end. A 1315 bp HindIII–PstI fragment from p35PPV-NAT (C terminus of NIB, entire CP and the 3′ ntr) was fused in-frame to the 3′ end of the gus gene by blunt-ending of the Xhol and HindIII sites. In addition, a 1726 bp BamHI–EcoRI fragment encoding most of NIB and the N terminus of the CP was fused in-frame to the 5′ end of the gus gene by blunt-ending the Ncol site. This cassette was reintroduced as a BamHI–Xhol fragment into p35PPV-NAT, leading to p35PPV-NAT-
Elimination of particle assembly in PPV

Fig. 1. (A) Assembly mutations and N-terminal deletion in the CP gene of PPV-NAT. Numbers indicate nucleotide positions in PPV-NAT according to Maiss et al. (1989). (B) Insertion of the β-glucuronidase gene (gus) into p35PPV-NAT. (C) Plant expression cassette for transformation of N. benthamiana line 17.27.4. (D) Plant expression cassette for transformation of N. benthamiana line 4.30.45 and constructs with mutated CP-NAT. NIa, Nuclear inclusion body a; NIb, nuclear inclusion body b; 3'-ntr, 3'-nontranslated region; pA, poly(A) tail; Q/A, NIa protease recognition sequence; pnos and nosA, nopaline synthase promoter and polyadenylation signal; p35S, 35S promoter from CaMV; gus, β-glucuronidase gene with intron; RB and LB, right and left borders of T-DNA.

gus-CP, thereby duplicating 27 aa of the CP and 18 aa of NIb, which remained as N- and C-terminal fusions, respectively, of the GUS protein. These duplicated sequences should allow proteolytic processing of the two viral proteins (Dougherty et al., 1988). The structure of the modified p35PPV-NAT is presented in Fig. 1. The resulting plasmid, p35PPV-NAT-gus-CP, was directly bombarded on N. benthamiana plants by using the particle inflow gun (PIG) (see below) to test its ability to replicate, recognition of the duplicated protease recognition sequences and its ability to produce systemic infections. The clone was mutated in the two assembly motifs (RQ and D) of the CP by exchanging part of the CP gene in p35PPV-NAT-gus-CP with the appropriate part from CP-RQ-D, resulting in p35PPV-NAT-gus-CP-RQ-D. Moreover, both plasmids were used for microprojectile bombardment on leaves of transgenic and non-transgenic N. benthamiana plants to detect GUS activity and virus particles (see below).

**Construction of pPVX-gus-Bsp120I.** A full-length cDNA clone of Potato virus X (PVX) under the control of the CaMV 35S promoter was kindly provided by D. C. Baulcombe and colleagues (pPVX201; Chapman et al., 1992). A gus gene was inserted under the control of the PVX CP promoter, resulting in pPVX-gus. This plasmid was subsequently linearized with Bsp120I, followed by a filling-in reaction with Klenov fragment and religation, generating an NgolI site. Successful introduction of a frame-shift into the M1 gene of the triple gene block (TGB) to render the virus movement-defective, as described by Morozov et al. (1997), was confirmed by restriction digestion of the resulting plasmid (pPVX-gus-Bsp120I). The resulting plasmid, pPVX-gus-Bsp120I, was tested for infectivity and the ability to form virus particles on leaves and systemic infections on whole plants of N. benthamiana by microprojectile bombardment (see below).

**Infectivity assay on whole plants with different p35PPV-NAT constructs.** Approximately 0.5–1 µg column-purified plasmid DNA (QIAGEN) of different p35PPV-NAT constructs was used for microprojectile bombardment on 4-week-old transgenic and non-transgenic N. benthamiana plants (four to six fully expanded leaves) by using the PIG (Gray et al., 1994). Systemic infection or complementation was confirmed by plate-trapped antigen (PTA)-ELISA (Hobbs et al., 1987) with antiserum to CP or, in the case of mutated CP, to helper component protease (HCpro), and electron microscopy (EM).

**Infectivity assay in N. benthamiana leaves with p35PPV-NAT-gus-CP constructs and pPVX-gus-Bsp120I.** Microprojectile bombardment on leaves of N. benthamiana plants was performed by using the flying disk method (Daniell, 1993) with the PDS-1000 particle gun (Biorad) as described by Morozov et al. (1997), except that the bombardment pulse was set to 1100 p.s.i. (approx. 7.6 MPa). Bombarded leaves were incubated for 72 h on moistened filter paper in a sealed Petri dish in the dark before the histochemical GUS assay was carried out (see below).

**Monitoring of replication and movement of different p35PPV-NAT-gus-CP constructs and PVX-gus-Bsp120I.** GUS
expression was monitored by histochemical detection, as described by Jefferson et al. (1987) and modified by De Block & Debrouwer (1992). Inoculated and incubated leaves were vacuum-infiltrated with the colorimetric GUS substrate 5-bromo-4-chloro-3-indolyl β-d-glucuronide (X-Gluc) at a concentration of 0.6 mg/ml in 0.1 M Na/KH₂PO₄ (pH 7.0); 10 mM EDTA, 3 mM K₃[Fe(CN)₆]. After overnight incubation at 37 °C, leaves were examined with a binocular microscope at 40× magnification without prior fixation in ethanol. Diameters of GUS foci were measured and analysed as described below.

- **Preparation of GUS foci for the detection of virus particles by ISEM.** Preparation of GUS foci for the detection of virus particles by ISEM was performed by cutting foci with a diameter of approximately 1–5 mm out of the leaves with a shortened Pasteur pipette. Ten of these GUS foci were ground in one drop of 0.1 M Na/KH₂PO₄ (pH 7.0) and subsequently used for ISEM preparations with CP-specific antiserum (see below).

- **Electron microscopy.** EM was carried out according to Mihne & Lesemann (1984). EM copper grids (400 mesh) with adsorbed virus particles were incubated with purified anti-PPV-NAT CP IgG (Riedel et al., 1998) in order to detect heterologous encapsidated potyvirus particles containing transgenic CP subunits from PPV. Heterologous encapsidated virions were detected by EM with goat anti-rabbit IgG (GaR) labelled with 10 nm gold particles (GaR-gold 10 nm). For ISEM, EM grids were coated with purified anti-PPV CP or anti-PVX CP IgG prior to incubation overnight on single drops of plant sap. Grids were incubated with the appropriate antiserum to decorate adsorbed virus particles.

- **Construction of plant expression vectors and Agrobacterium-mediated plant transformation.** Plants were transformed with mutated CP genes from pe35SL-CP-NAT, which was inserted together with a gus gene (Vancanneyt et al., 1990) into the binary vector pLX222. The vector delivers the transgenic CP subunits from PPV. Heterologous encapsidated transgenic CP was first described by Lindbo & Dougherty (1992) in order to detect heterologous encapsidated potyvirus particles containing transgenic CP subunits from PPV. Heterologous encapsidated virions were detected by EM using goat anti-rabbit IgG (GaR) labelled with 10 nm gold particles (GaR-gold 10 nm). For ISEM, EM grids were coated with purified anti-PPV CP or anti-PVX CP IgG prior to incubation overnight on single drops of plant sap. Grids were incubated with the appropriate antiserum to decorate adsorbed virus particles.

- **Selection and analysis of homozygous PPV-resistant T₂ N. benthamiana plants.** Regenerated plants were selfed and T₁ seeds were produced. Four-week-old T₁ plants were inoculated with PPV-NAT. Lines resistant to PPV were selected for subsequent production of homozygous T₂ seeds. Homozygous lines were identified by germinating kanamycin-containing MS agar (Murashige & Skoog, 1962).

- **Selection of transgenic CP expression lines and Agrobacterium-mediated plant transformation.** Plants were transformed with mutated CP genes from pe35SL-CP-NAT, which was inserted together with a gus gene (Vancanneyt et al., 1990) into the binary vector pLX222. The vector delivers the transgenic CP subunits from PPV. Heterologous encapsidated virions were detected by EM using goat anti-rabbit IgG (GaR) labelled with 10 nm gold particles (GaR-gold 10 nm). For ISEM, EM grids were coated with purified anti-PPV CP or anti-PVX CP IgG prior to incubation overnight on single drops of plant sap. Grids were incubated with the appropriate antiserum to decorate adsorbed virus particles.

- **Preparation of GUS foci for the detection of virus particles by ISEM.** Preparation of GUS foci for the detection of virus particles by ISEM was performed by cutting foci with a diameter of approximately 1–5 mm out of the leaves with a shortened Pasteur pipette. Ten of these GUS foci were ground in one drop of 0.1 M Na/KH₂PO₄ (pH 7.0) and subsequently used for ISEM preparations with CP-specific antiserum (see below).

- **Electron microscopy.** EM was carried out according to Mihne & Lesemann (1984). EM copper grids (400 mesh) with adsorbed virus particles were incubated with purified anti-PPV-NAT CP IgG (Riedel et al., 1998) in order to detect heterologous encapsidated potyvirus particles containing transgenic CP subunits from PPV. Heterologous encapsidated virions were detected by EM with goat anti-rabbit IgG (GaR) labelled with 10 nm gold particles (GaR-gold 10 nm). For ISEM, EM grids were coated with purified anti-PPV CP or anti-PVX CP IgG prior to incubation overnight on single drops of plant sap. Grids were incubated with the appropriate antiserum to decorate adsorbed virus particles.

- **Construction of plant expression vectors and Agrobacterium-mediated plant transformation.** Plants were transformed with mutated CP genes from pe35SL-CP-NAT, which was inserted together with a gus gene (Vancanneyt et al., 1990) into the binary vector pLX222. The vector delivers the transgenic CP subunits from PPV. Heterologous encapsidated virions were detected by EM using goat anti-rabbit IgG (GaR) labelled with 10 nm gold particles (GaR-gold 10 nm). For ISEM, EM grids were coated with purified anti-PPV CP or anti-PVX CP IgG prior to incubation overnight on single drops of plant sap. Grids were incubated with the appropriate antiserum to decorate adsorbed virus particles.

- **Selection and analysis of homozygous PPV-resistant T₂ N. benthamiana plants.** Regenerated plants were selfed and T₁ seeds were produced. Four-week-old T₁ plants were inoculated with PPV-NAT. Lines resistant to PPV were selected for subsequent production of homozygous T₂ seeds. Homozygous lines were identified by germinating kanamycin-containing MS agar (Murashige & Skoog, 1962).

- **The number of transgene insertions was determined by Southern hybridization with DIG chemiluminescence detection (Boehringer) using a 35S promoter probe.**

- **Transgene expression of the CP was determined after SDS-PAGE and Western blot (Towbin et al., 1979) of total plant protein extracts (Berger et al., 1989) followed by immunoassay with purified IgG against PPV-NAT CP.**

- **Transgenic N. benthamiana lines expressing functional CP of PPV-AT or PPV-NAT.** Two transgenic N. benthamiana lines were used in complementation experiments and as positive controls in the heterologous encapsidation experiments. One of the transgenic T₃ lines (17.27.4) expresses a single copy of the functional CP of the aphid-transmissible strain of PPV (PPV-AT) in a tandem array with the 36 C-terminal amino acid residues from NbCP (Timpe et al., 1992). Only 54 bp of the PPV 3'-ntr were included in this construct (Fig. 1C). The line displays recovery resistance when infected with PPV. This phenomenon was first described by Lindbo & Dougherty (1992b). The other homozygous transgenic N. benthamiana T₃ line (4.30.45) was transformed with the CP gene of PPV-NAT. The plant expression cassette contains a complete PPV 3'-ntr (Korte et al., 1995) (Fig. 1D). The arrangement of the different genes in the plant expression cassette was the same as for the constructs containing mutated CP genes. This line, containing one copy of the transgene, displayed the same recovery-resistance phenomenon when infected with PPV.

- **Statistical analysis.** Numbers of gold-decorated virus particles and diameters of GUS foci were analysed by using General Linear Models (GLM). Multiple mean comparisons were computed by using the Dunnet test (SAS Institute, 1996).

### Results

**Effect of CP mutations on infectivity of p35PPV-NAT in non-transgenic and transgenic N. benthamiana plants expressing functional PPV CP**

Each of the mutated full-length clones, with a mutation in either the RQ or D motif or mutations in both motifs (p35PPV-NAT-RQ, -D and -RQ-D), was bombarded in three independent experiments on non-transgenic N. benthamiana plants (five plants per experiment), by using the pIG, in order to test their ability to produce systemic infections. Only p35PPV-NAT, which was bombarded as a positive control, infected non-transgenic N. benthamiana plants, at a rate of 80–100%. PTA-ELISA with antiserum to HCpro revealed no PPV infection in plants bombarded with the CP-mutated full-length clones. All clones with the reintroduced wild-type PPV-NAT CP sequence displayed systemic infections in non-transgenic N. benthamiana, indicating that mutations in the assembly motifs were solely responsible for the absence of systemic infection.

All three mutants of assembly motifs of p35PPV-NAT CP were subsequently tested for systemic infection in the transgenic line 17.27.4. The functional transgenic CP complemented the defective CP of the virus and allowed the mutants to infect plants systemically. Symptoms appeared 12–16 days after inoculation, but remained very mild compared with wild-type PPV symptoms, which were already visible 6–8 days after inoculation. Only local leaf clearing, and no leaf rolling or epinasty, was observed. The infection rate of the mutants by microprojectile bombardment was substantially lower than that of the wild-type PPV, however. It varied between one and three plants infected of five plants inoculated in three independent infectivity assays. Despite complementation of the mutation in the viral sequence, recovery of newly formed leaves from symptoms was determined visually 3–4 weeks after inoculation, as is the case in infection with wild-type PPV (Timpe et al., 1992). Systemic infections of all three mutants were monitored by PTA-ELISA with antiserum to HCpro and displayed ELISA readings comparable to transgenic plants infected with parental virus (data not shown). In contrast, only very few virus particles were detected in ISEM preparations of systemically infected leaves. Ten to twenty fields of view (each 1600 μm²) on one grid had to be examined by EM to detect one virus particle.

It was necessary to test whether the virus assembly-
demonstrating that cell-to-cell movement was inhibited by the gus fold smaller than those produced by unmodified PPV- and cell-to-cell movement, the plasmids p35PPV-NAT-GUS. The foci of assembly-defective PPV-

ment of the virus was monitored by histochemical analysis of leaves by using the PDS-1000 (Table 1). Move-

benthamiana

leaves expressing a functional PPV CP

benthamiana

line 17.27.4, infected with the different assembly

transgenic CP or had been restored by recombination with defective CP was simply complemented by the functional CP.
The foci of assembly-defective PPV-

mended p35PPV-NAT-

leaves. However, the spots were significantly smaller than those produced by p35PPV-NAT-gus-CP in non-transgenic

benthamiana leaves. However, the spots were significantly smaller than those produced by p35PPV-NAT-gus-CP in non-transgenic

benthamiana leaves (Table 1). This indicates that cell-to-cell movement functions were only partially restored by the transgenic CP. GUS foci of all four treatments (p35PPV-NAT-gus-CP, p35PPV-NAT-gus-CP-RQ-D, complemented p35PPV-NAT-gus-CP-RQ-D and pPVX-gus-Bsp120I) were prepared for ISEM, followed by decoration with CP-specific antisera. pPVX-gus-Bsp120I was used as a movement-defective but assembly-intact control. Four grids were prepared for each treatment and 60 or 120 fields of view of each grid were checked by EM for the presence of virus particles (Table 1). The number of virus particles was counted in each field. The detection of virus particles in GUS foci of movement-defective PVX-gus showed that the sensitivity of the assay was high enough to detect particles in GUS foci of single infected cells. Virus particles were only found in GUS foci produced from unmodified p35PPV-NAT-gus-CP or from p35PPV-NAT-gus-CP-RQ-D complemented with the transgenic CP. In contrast, no virus particles could be detected in GUS foci of assembly-defeated p35PPV-NAT-gus-CP from N. benthamiana leaves, even though twice the number of fields of view were examined on each grid. This demonstrates that the mutation of both assembly motifs (RQ and D) in the CP not only inhibited cell-to-cell movement but also inhibited virion assembly of PPV.

**Effect of mutated CPs on heterologous encapsidation with challenging viruses in transgenic plants**

Four to ten transgenic T3 lines of each construct were characterized for resistance. Homozygous lines were used for

<table>
<thead>
<tr>
<th>Clone</th>
<th>Leaves inoculated</th>
<th>GUS foci counted (n)</th>
<th>Focus diameter (µm)*</th>
<th>Grids prepared (n)</th>
<th>No. and area (µm²) of fields examined</th>
<th>Particles per field (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p35PPV-NAT-gus-CP</td>
<td>N. b.</td>
<td>217</td>
<td>746 ± 10*</td>
<td>4</td>
<td>60 (96000)</td>
<td>93.8 ± 22.8</td>
</tr>
<tr>
<td>p35PPV-NAT-gus-CP-RQ-D</td>
<td>N. b.</td>
<td>116</td>
<td>155.7 ± 16*</td>
<td>4</td>
<td>120 (192000)</td>
<td>1.9 ± 1.5</td>
</tr>
<tr>
<td>p35PPV-NAT-gus-CP-RQ-D</td>
<td>N. b. (transgenic)</td>
<td>200</td>
<td>517.5 ± 5.1*</td>
<td>4</td>
<td>60 (96000)</td>
<td>14.2 ± 13.0</td>
</tr>
<tr>
<td>pPVX-gus-Bsp120I</td>
<td>N. b.</td>
<td>191</td>
<td>149.2 ± 26*</td>
<td>4</td>
<td>60 (96000)</td>
<td></td>
</tr>
</tbody>
</table>

* Values followed by the same letter are not significantly different (P > 0.001).

defective CP was simply complemented by the functional transgenic CP or had been restored by recombination with transgenic transcripts. Therefore, plant sap from transgenic N. benthamiana line 17.27.4, infected with the different assembly mutants, was inoculated on five non-transgenic N. benthamiana plants. In the case of recombination, the wild-type sequence could have been restored, leading to a systemic infection of the non-transgenic N. benthamiana plants. In three independent experiments, five successive passages from the transgenic N. benthamiana line 17.27.4 to non-transgenic N. benthamiana plants were carried out. In all experiments, none of the inoculated plants displayed a systemic infection, indicating that no recombination had occurred. In order to confirm successful transmission and complementation on transgenic N. benthamiana plants, plants of line 17.27.4 were inoculated at the same time. These transgenic plants developed systemic symptoms and showed complementation as described earlier.

**Effect of the CP-RQ-D mutation on particle assembly of p35PPV-NAT in non-transgenic and transgenic N. benthamiana leaves expressing a functional PPV CP**

The full-length PPV clone, engineered to express the gus gene (p35PPV-NAT-gus-CP), was bombarded on N. benthamiana plants by using the PIG. Typical PPV symptoms appeared 5–7 days after inoculation and histochemical GUS analysis of systemically infected leaves subsequently revealed the presence of active GUS. This indicated processing of Nlβ and CP from p35PPV-NAT-gus-CP and the presence of a functional GUS despite the short N- and C-terminal fusions.

In order to demonstrate in planta that the two CP assembly motifs (RQ and D) of PPV were involved in particle assembly and cell-to-cell movement, the plasmids p35PPV-NAT-gus-CP and p35PPV-NAT-gus-CP-RQ-D were bombarded on N. benthamiana leaves by using the PDS-1000 (Table 1). Movement of the virus was monitored by histochemical analysis of GUS. The foci of assembly-defective PPV-gus were about 4.5-fold smaller than those produced by unmodified PPV-gus, demonstrating that cell-to-cell movement was inhibited by the mutated CP (RQ-D). The diameters of the blue foci were comparable to those produced by pPVX-gus-Bsp120I, which was used as a movement-defective control. To test for trans-complementation of the movement defect, p35PPV-NAT-gus-CP-RQ-D was bombarded on transgenic N. benthamiana 17.27.4 plants expressing the intact PPV CP. Subsequent GUS staining revealed foci with diameters that were significantly greater (P < 0.0001) than those produced in non-transgenic N. benthamiana leaves. However, the spots were significantly smaller than those produced by p35PPV-NAT-gus-CP in non-transgenic N. benthamiana leaves (Table 1). This indicates that cell-to-cell movement functions were only partially restored by the transgenic CP. GUS foci of all four treatments (p35PPV-NAT-gus-CP, p35PPV-NAT-gus-CP-RQ-D, complemented p35PPV-NAT-gus-CP-RQ-D and pPVX-gus-Bsp120I) were prepared for ISEM, followed by decoration with CP-specific antisera. pPVX-gus-Bsp120I was used as a movement-defective but assembly-intact control. Four grids were prepared for each treatment and 60 or 120 fields of view of each grid were checked by EM for the presence of virus particles (Table 1). The number of virus particles was counted in each field. The detection of virus particles in GUS foci of movement-defective PVX-gus showed that the sensitivity of the assay was high enough to detect particles in GUS foci of single infected cells. Virus particles were only found in GUS foci produced from unmodified p35PPV-NAT-gus-CP or from p35PPV-NAT-gus-CP-RQ-D complemented with the transgenic CP. In contrast, no virus particles could be detected in GUS foci of assembly-defeated p35PPV-NAT-gus-CP from N. benthamiana leaves, even though twice the number of fields of view were examined on each grid. This demonstrates that the mutation of both assembly motifs (RQ and D) in the CP not only inhibited cell-to-cell movement but also inhibited virion assembly of PPV.
Table 2. Attributes of selected transgenic T₂ lines expressing the different mutated PPV-NAT CP genes
The mean numbers of heterologous encapsidated ChiVMV and PVY particles detected with transgenic PPV CPs are shown.

<table>
<thead>
<tr>
<th>T₂ line</th>
<th>CP gene</th>
<th>Copy number</th>
<th>Protein expression</th>
<th>Resistance type*</th>
<th>ChiVMV†</th>
<th>PVY-N†</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. benthamiana</td>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>53-4a</td>
<td>10-2a</td>
</tr>
<tr>
<td>17.27.4</td>
<td>CP-AT‡</td>
<td>1§</td>
<td>+</td>
<td>r</td>
<td>181-0b</td>
<td>194-4b</td>
</tr>
<tr>
<td>4.30.45</td>
<td>CP-NAT</td>
<td>1</td>
<td>+</td>
<td>r</td>
<td>163-0b</td>
<td>197-4b</td>
</tr>
<tr>
<td>7.14.2</td>
<td>CP-RQ</td>
<td>2</td>
<td>+</td>
<td>r</td>
<td>81-8b</td>
<td>9-8b</td>
</tr>
<tr>
<td>21.16.2</td>
<td>CP-D</td>
<td>2</td>
<td>+</td>
<td>i</td>
<td>42-0b</td>
<td>7-8b</td>
</tr>
<tr>
<td>19.8.6</td>
<td>CP-RQ-D</td>
<td>1</td>
<td>+</td>
<td>i</td>
<td>40-6a</td>
<td>15-4a</td>
</tr>
</tbody>
</table>

* Resistance types shown are: r, recovery resistance (plants recover from systemic infection 3–4 weeks post-inoculation), and i, infection resistance (plants could not be infected by mechanical inoculation).
† Number of particles decorated per 200 particles counted from five different plants. Means followed by the same letters are not significantly different (P > 0.0001). Decoration was with anti-PPV CP IgG and GaR–gold 10 nm.
‡ No mutation, 36 C-terminal amino acids (from NIb) added.
§ A single insertion of a tandem repeat (see Fig. 1C).

Detection of CP expression by Western blot analysis. Table 2 shows the plant lines selected for examination of heterologous encapsidation with different challenging viruses. Chilli veinal mottle virus (ChiVMV), PVY (both potyviruses) and TMV (a tobamovirus) were used for inoculation. After the appearance of systemic symptoms, 200 particles of TMV from one plant and 200 particles of ChiVMV and PVY from five plants were analysed for heterologous encapsidation. For TMV, only two (17.27.4) and four (7.14.2) viirons of 200 examined were gold-decorated (data not shown), indicating that no heterologous encapsidation had occurred. ChiVMV and PVY infection in lines 17.27.4 and 4.30.45 yielded up to 100% decoration (10–15 gold particles per virion). The other lines tested, containing the three different mutated CPs, did not show significant heterologous encapsidation compared with non-transgenic N. benthamiana plants (Table 2). Virus particles from these plants, as well as from non-transgenic plants, were mainly undecorated or showed only one to three gold particles on the surface.

Suppression of complementation of p35PPV-NAT mutants in transgenic lines expressing dysfunctional PPV CP

In order to demonstrate that assembly-defective CPs expressed in transgenic N. benthamiana lines were not able to complement assembly-defective PPV mutants, different transgenic lines were used for infectivity assays with p35PPV-NAT-RQ, -D and -RQ-D. Five plants of each transgenic recovery-resistant T₂ line were bombarded with the three different assembly-defective p35PPV-NAT constructs. The number of systemically infected plants was determined visually and subsequently confirmed by ELISA to HCpro and compared with control line 17.27.4 and non-transgenic N. benthamiana plants. No systemic infection or complementation was found in the transgenic lines expressing the mutated CP genes.

Discussion

Mutations in the CP (-RQ, -D and -RQ-D) were introduced into a full-length clone of PPV-NAT in order to determine their effect on virion formation, replication and cell-to-cell movement. Assays on whole plants with different mutants of p35PPV-NAT containing dysfunctional CPs resulted in no systemic infection. It cannot be concluded whether the inability to produce systemic infection was due to the absence of replication or of movement. Complementation and systemic infection of all three mutants in transgenic functional PPV CP-expressing N. benthamiana plants showed that the mutations in the CP gene did not inhibit replication of the viral genome. If one of the mutations in the CP gene of PPV had prevented initiation of replication of the viral genome, it would not have been possible to detect complementation by an intact transgenic CP or CP gene. ELISA readings with antiserum to HCpro contradicted the very small numbers of virus particles detected by EM compared with wild-type PPV infection of 17.27.4 plants. The concentration of functional transgenic CP possibly limited encapsidation. Dolja et al. (1994) failed to detect virions in similar experiments with assembly-mutated TEV-gus in CP-transgenic plants and protoplasts. This could be due to a very low concentration of transgenic CP, insufficient to assemble into virions. Therefore, the question of whether the dys-
functional CP inhibited virion assembly and/or cell-to-cell movement remains.

Virus functions can be monitored by inserting reporter genes such as gus into infectious full-length clones (for a review see Scholthof et al., 1996). Therefore, a gus gene was introduced into PPV-NAT between the Nb and CP genes. This is an alternative to the previous strategy for marker-gene insertion into the genome of a potyvirus (Dolja et al., 1992), where GUS was produced as a fusion protein together with the HCpro of TEV. In a modified construct of TEV-gus, Schaad et al. (1997) introduced an additional Nla proteolytic cleavage site with the 6 kDa protein (6K<sub>N</sub>) between the gus and HCpro genes to release the reporter protein from HCpro. Our approach shows that it is also possible to use a duplicated Nb–CP proteolytic cleavage site with short adjacent sequences of the Nla and CP genes. Obviously, different regions in the potyviral genome would also be suitable for the insertion of a foreign gene. The construct offers the advantage that GUS is released from the viral polyprotein with minimal fusions at the N and C termini. Experiments to test the stability of the chimeric PPV-gus after mechanical transmission are in progress.

Sensitive detection of GUS-tagged PVX, even in single infected cells, was demonstrated with ISEM assays directly from GUS-stained infection foci. This enables studies of virus assembly and replication in planta without being obliged to produce protoplasts. The results of the infectivity assays, the GUS analysis and the ISEM-detection of p35PPV-NAT-gus CP and CP-RQ-D from transgenic leaves provide direct evidence that the amino acids RQ and D of PPV CP are involved in virion formation in planta. Only when wild-type CP was translated from the viral genome or supplied in trans from transgenic plants was virion assembly observed. Mutations to amino acids with differing attributes in these motifs disrupted particle assembly, confirming the earlier results of Jagdish et al. (1991, 1993) and Jacquet et al. (1998a), who gained similar results with the expression of wild-type and assembly-mutated potyvirus CP in E. coli or Saccharomyces cerevisiae. Interestingly, assembly was detected in the absence of the entire viral RNA in these artificial systems. However, foreign expression systems revealed similar results to those obtained by the in planta system presented here. Virions produced from assembly-defective PPV-gus were detected in GUS foci 3 days post-inoculation from transgenic N. benthamiana 17.27.4 leaves with a mean number of about two particles per field of view (Table 1). Several times fewer virions could be detected 14 days post-inoculation in transgenic N. benthamiana 17.27.4 plants infected systemically with each of the assembly-mutated p35PPV-NAT constructs. A possible explanation is the degradation of viral RNA due to the recovery of transgenic plants inoculated with the assembly-defective mutants, leading to a smaller number of virus particles.

Only the double mutant was tested for its influence on assembly. Hence, it is not clear whether either of the mutations alone is able to suppress particle formation. Dolja et al. (1994) demonstrated that this occurs for TEV-gus in their experiments with single CP mutations (RQ or D). The non-occurrence of heterologous encapsidation with challenging potyviruses in the transgenic N. benthamiana lines containing CP with single mutations in the RQ or D motif provides indirect evidence that each of the mutated motifs alone can also abolish potyvirus virion assembly.

Not only virion assembly but also cell-to-cell movement was abolished by the RQ and D mutation in the PPV CP. The movement function was only partially restored by the transgenic CP, as shown by the smaller GUS foci produced from PPV-gus-CP-RQ-D in transgenic leaves (Table 1). The formation of significantly smaller spots might be due to a limited concentration of the transgenic CP, which might limit or delay virus movement. Our results therefore support the previous findings of Dolja et al. (1994) with assembly mutants of TEV-gus, except the detection of virions in transgenic plants or leaves infected with assembly mutants.

To date, it is not clear whether potyviruses move from cell to cell as virions or as non-virion ribonucleoproteins. According to Dolja et al. (1994), the lack of detectable virions in transgenic plants infected with the assembly mutant of TEV-gus may support the fact that the CP has a transport-facilitating role that is different from its role in encapsidation. Rodriguez-Cerezo et al. (1997) found CP complexes with a linear shape inside or attached to cylindrical inclusion proteins (CI) near plasmodesmatal connections in ultrathin sections of mesophyll cells of tobacco leaves infected with Tobacco vein mottling virus. These authors suggested that these complexes could be virions that were targeted for transport, but were not able to exclude the possibility of a non-virion ribonucleoprotein transport complex. In similar labelling experiments with PPV, Riedel et al. (1998) could not distinguish whether CP filaments within plasmodesmata were virions or merely CP aggregates. The occurrence of cell-to-cell movement only in the presence of virus particles in our experimental system supports the hypothesis of cell-to-cell transport of virions. The two CP functions of assembly and transport cannot be separated. A modification or deletion of the origin of assembly, probably located at the 5’ end (Wu & Shaw, 1998), may possibly help to determine the form of cell-to-cell transport of potyviruses.

Our results indicate that resistance of transgenic plants to a potyvirus is not influenced by local mutations in the transgenic CP of PPV (CP-RQ-D or -RQ-D). This strategy of rendering a CP gene dysfunctional but suitable for production of resistance could eventually be applied to other viruses that also contain the postulated salt bridge between the conserved R and D residues in the CP (Dolja et al., 1991).

In recent years, many agronomically important CP-transgenic cultivars have been tested in small-scale field trials. Commercial virus-resistant transgenic squash and papaya varieties are available in the USA (White, 1999). In these cases, complete and functional genes were used for plant transformation. Although potential biological risks (e.g. comple-
mentation, recombination, heterologous encapsidation and synergism) in virus-resistant transgenic plants could be demonstrated in case studies, so far no biological hazards have been observed in field releases. However, recent studies provide more information about multiple functions of viral proteins. This offers the possibility of modifying defined motifs within a gene, related to specific functions of a protein.

In transgenic Nicotiana benthamiana plants with modified PPV CP genes, heterologous encapsidation with potyviruses can be effectively suppressed. In addition, the trans-complementation of CP functions of challenging mutants of PPV was completely abolished in the plant lines that expressed dysfunctional CPs.

The transgenic constructs characterized in this study offer an additional possibility for the use of untranslatable viral genes or gene fragments for the production of virus-resistant transgenic plants, independent of the kind of inherent resistance mechanism. Modifications can be introduced as easily as stop codons into viral genes. However, non-translatable or antisense viral gene mediate virus resistance without providing protein functions to challenging viruses. By using wild-type genes for non-translatable constructs, virus functions could nevertheless be transferred to infecting viruses through recombination processes (Greene & Allison, 1994; Borja et al., 1999). To minimize even putative biological risks of virus-resistant transgenic plants, it would therefore be advantageous to combine these two approaches. An untranslatable viral gene in transgenic plants assures that no additional transgenic protein is added to the plant. In addition, the removal of specific functions from a viral protein by mutation of the corresponding gene can abolish the transfer of functions to challenging viruses via recombination.

We thank Dr D. C. Baulcombe and his colleagues at the Sainsbury Laboratory for generously providing the plant expression plasmid pPVX201, Dr J. Landsmann for providing pLX222 and J. Zimmermann for excellent technical assistance. M. Varrelmann was funded by GIF contract no. 1-307-141.12/93.

References


Received 19 July 1999; Accepted 29 October 1999