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Transgenic or Plant Expression Vector-Mediated Recombination of *Plum Pox Virus*

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Different mutants of an infectious full-length clone (p35PPV-NAT) of *Plum pox virus* (PPV) were constructed: three mutants with mutations of the assembly motifs RQ and DF in the coat protein gene (CP) and two CP chimeras with exchanges in the CP core region of *Zucchini yellow mosaic virus* and *Potato virus Y*. The assembly mutants were restricted to single infected cells, whereas the PPV chimeras were able to produce systemic infections in *Nicotiana benthamiana* plants. After passages in different transgenic *N. benthamiana* plants expressing the PPV CP gene with a complete (plant line 4.30.45.) or partially deleted 3′-nontranslated region (3′-NTR) (plant line 17.27.4.), characterization of the viral progeny of all mutants revealed restoration of wild-type virus by recombination with the transgenic CP RNA only in the presence of the complete 3′-NTR (4.30.45.). Reconstitution of wild-type virus was also observed following cobombardment of different assembly-defective p35PPV-NAT together with a movement-defective plant expression vector of *Potato virus X* expressing the intact PPV-NAT CP gene transiently in nontransgenic *N. benthamiana* plants. Finally, a chimeric recombinant virus was detected after cobombardment of defective p35PPV-NAT with a plant expression vector-derived CP gene from the sour cherry isolate of PPV (PPV-SoC). This chimeric virus has been established by a double recombination event between the CP-defective PPV mutant and the intact PPV-SoC CP gene. These results demonstrate that viral sequences can be tested for recombination events without the necessity for producing transgenic plants.

It is generally assumed that RNA recombination is one of the major driving forces in the evolution of plant viruses. This process leads to rearrangement of viral genomes and exchange of specific modular functions among different viruses (1, 3, 9, 26, 35, 43, 47). Together with the high mutation rate resulting from the low proofreading activity of viral RNA-dependent RNA polymerases (RdRp) (10, 48) and in combination with short replication cycles. RNA recombination plays an important role in adaptation, genome repair, and genetic variability of RNA viruses. Several models for RNA recombination have been proposed, but nearly all available evidence supports an RdRp-mediated template switch model in which recombination occurs during RNA synthesis (1, 35).

Recombination has been observed in several plant virus genera, e.g., in *Bromovirus*, where genetic recombination with RNA3 deletion mutants of *Brome mosaic virus* has been shown, in *Alfamovirus* between deletion mutants of *Alfalfa mosaic virus* (51), and in *Cucumovirus* (12). Recombination events in nepoviruses were also detected with the use of pseudorecombinant isolates (28). In addition, recombinants with nonreplicating viral RNA components have been detected for 3′-truncated viral and defective interfering RNAs of different tombusviruses (55).

Other types of evidence of viral RNA recombination are based on phylogenetic analysis of RNA viral genomes (12, 17, 27, 30, 41, 44). Recently, experimental evidence for recombination among potyviruses between two artificially produced defective RNA species of *Zucchini yellow mosaic virus* (ZYMV) was presented (16). Recombination events have been proposed, based on comparisons of sequences of different naturally occurring strains of potyviruses, including *Plum pox virus* (PPV) (6, 39).

When a viral genome segment is expressed in transgenic plants to mediate virus resistance, the entire transcript or a portion of the transcript can, under certain circumstances, be incorporated into the genome of a challenging virus by means of recombination. Concerns have been raised about the evolution of new viruses with altered virulence, host range, or vector specificity by recombination in transgenic plants (8, 24, 42, 45, 49). Recently, several recombination events have been demonstrated in transgenic plants expressing viral genes. Most of the examples (14, 15, 19, 29, 45) were found under conditions of high selection pressure; e.g., the viral inoculum used for challenging of plants was rendered movement defective. In two more recent studies, recombinant viruses were isolated from transgenic plants under conditions of moderate selection pressure. The recombinant progeny viruses had a distinct competitive advantage compared to the parental inoculum, which was able to infect the plants systemically (4, 56). However, there are no experimental data for recombination between potyvirus transgenes and mutants of the corresponding parental virus.

The model system used in our experiments is PPV in *Nicotiana benthamiana*. PPV, the causal agent of sharka disease, which affects stone fruit trees, belongs to the *Potyviridae* family, the largest family of plant viruses, whose members cause severe diseases in a number of economically important crops.

One of the main objectives of our study was to investigate whether recombination events in transgenic plants containing a PPV coat protein (CP) gene can rescue a potyvirus mutant defective in coat protein assembly. This was achieved by introducing assembly mutations of the CP gene into an infectious full-length clone of PPV (p35PPV-NAT) (32). These mutations exerted a strong selection pressure in the recombination experiments because the initial mutant was unable to system-

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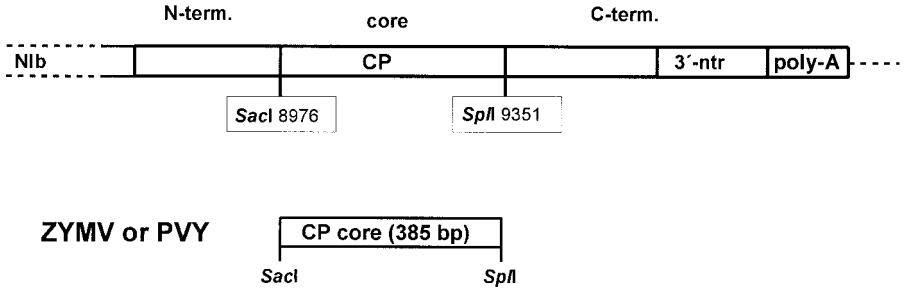

FIG. 1. Replacement of the CP core region in p35PPV-NAT with the appropriate part of ZYMV or PVY. Numbers indicate nucleotide positions in PPV-NAT and are those used in reference 31.

ically infect nontransgenic *N. benthamiana* plants (52). As described above, recombinant viruses can be isolated from transgenic plants, even when the initial virus is able to infect the plants systemically. For this purpose, the highly conserved core region of the PPV CP gene was replaced with the corresponding region of two other potyviruses, ZYMV and *Potato virus Y* (PVY). The ability of the mutants to induce systemic infections in *N. benthamiana* plants and the suitability for recombination experiments under moderate or low selection pressure was examined. All different CP mutants were also tested for recombination on transgenic *N. benthamiana* plants expressing the functional CP of PPV.

Additionally, a strategy was developed to reduce the possibility of recombination events by modifying the transgenic sequence. Greene and Allison (20) detected recombinant viruses only when the transgene had no deletions in the 3′-nontranslated region (3′-NTR) adjacent to the CP of *Cowpea chlorotic mottle bromovirus* (CCMV). The authors concluded that replication initiation functions of the 3′-NTR (13) and a possible reduced transgenic target length might be involved in prevention of recombination. Therefore, it was suggested that replication initiation sites be excluded from transgene constructions (2). On the basis of these findings, we investigated whether the 3′-NTR of the potyvirus genome, which is involved in replication initiation as well (21), plays a similar role in RNA recombination in transgenic plants expressing potyvirus sequences.

A further objective of the experiments was to reproduce recombinations in transgenic plants with an artificial system to investigate the likelihood of recombinations prior to producing transgenic plants. A valid model system would allow one to test different virus-derived sequences for the ability to recombine before starting time- and labor-intensive plant transformation experiments. On the other hand, it could be used as a tool to determine the preferential junction sites in recombination of potyviruses as well as of other viruses.

Instead of expression as a transgene, the PPV CP sequence should be transiently provided by a *Potato virus X* (PVX)-derived plant expression vector (pPVX201) (7). Recombinant PVX expression vector and different PPV CP mutants were used for cobombardment recombination assays to restore intact PPV.

In this study, we observed for the first time recombination of a potyvirus in transgenic plants transformed with the corresponding functional CP gene. In addition, we were able to detect potyvirus recombination events when a functional CP gene was expressed from a plant expression vector in nontransgenic plants. Finally, recombination was also detected with a coexpressed CP gene of a related PPV isolate and thereby allowed identification of the recombination sites.

MATERIALS AND METHODS

Site-directed mutagenesis of the PPV-NAT CP assembly motifs and replacement in p35PPV-NAT. The CP gene of PPV-NAT, subcloned into a plant expression vector, was modified using PCR mutagenesis as previously described (52). The different mutated parts of the CP gene were introduced into p35PPV-NAT using restriction sites *Sac*I and *Pst*I, which resulted in p35PPV-NAT-CP-RQ, p35PPV-NAT-CP-DF, and p35PPV-NAT-CP-RQ-DF. All mutations abolished particle assembly and systemic movement of the virus, but a functional transgenic PPV CP complemented the defective CP functions as described previously (52).

Construction of CP-chimeric p35PPV-NAT. To amplify the core region of ZYMV and PVY CP and simultaneously introduce the appropriate restriction sites (*Sac*I-*Spl*I) for subcloning into p35PPV-NAT, total RNA was extracted from ZYMV-infected *Cucumis sativus* and PVY-infected *N. benthamiana* (53) and used in reverse transcription-PCR (RT-PCR) by standard methods involving avian myeloblastosis virus reverse transcriptase (Invitrogen) and *Taq* polymerase (MBI) with the primers ZYMV-up (5′-ACACGAGCTCCTCATCAGCAGTTC GCCTC-3′), ZYMV-low (5′-CGGATCCGTACGGGAGTTTTAGAATTGAC T-3′), PVY-up (5′-AGAGCTCCTCAATCACAGTTT G-3′), and PVY-low (5′-CCGTACGGGTGTTCGTGATG-3′) (underlined letters display restriction recognition sequences). The resulting PCR fragments were subcloned into the plasmid pBluescriptII (SK−) (Stratagene), sequenced, and subsequently introduced into p35PPV-NAT with *Sac*I and *Spl*I, resulting in p35PPV-CP-ZYMV and p35PPV-CP-PVY (Fig. 1). The two chimeric constructs were used for infectivity assays in transgenic and nontransgenic *N. benthamiana* plants.

PVX-derived plant expression vector. A virus-derived plant expression vector based on PVX under control of the *Cauliflower mosaic virus* CaMV 35S promoter for the production of in vivo transcripts (pPVX201) was kindly provided by D. C. Baulcombe (7). This vector allows the expression of introduced foreign genes under control of the duplicated subgenomic CP promoter (Fig. 2).

Construction of movement-defective pPVX201. To construct a movement-defective mutant of pPVX201 (34, 38), a frameshift mutation was introduced into the M1 gene of the triple-gene block by linearizing a *Bsp*120I site (position 4946), filling with Klenow fragment, and religating to generate an *Ngo*MIV site. Successful mutagenesis was confirmed by restriction digestion of the resulting plasmid (pPVX201-*Bsp*120I). Triple-gene block-defective pPVX201-*Bsp*120I was used to bombard *N. benthamiana* plants to examine its capacity to cause systemic infections.

Introduction of CP genes of different PPV strains into pPVX201-*Bsp*120I. The CP genes of PPV-NAT and the sour cherry-infecting PPV isolate (PPV-SoC) were introduced into pPVX-*Bsp*120I (Fig. 2). CP-NAT from the plasmid pe35SL-NAT-CP was transferred together with a start codon (AUG), a complete 3′-NTR, a poly(A) tail, and a polyadenylation signal from CaMV to pPVX201-*Bsp*120I using the restriction sites *Cla*I and *Sal*I. The coding sequence of CP-SoC including nucleotides 115 to 1360 (36) (EMBL accession no. X97398) containing the 5′-terminal 30 bp of the NIb gene, the complete 3′-NTR, and a poly(A) tail was inserted as a *Cla*I-*Xho*I fragment into the plant expression vector. The resulting clones were named pPVX201-*Bsp*120I-CP-NAT and pPVX201-*Bsp*120I-CP-SOC, respectively. The different PVX-derived constructs were used, together with assembly mutants of p35PPV-NAT, to cobombard nontransgenic *N. benthamiana* plants. The PPV-NAT CP was also inserted into unmodified pPVX to obtain a positive control for particle bombardment assays.

Infectivity assay with p35PPV-NAT and pPVX constructs. Approximately 0.5 to 1 μg of column-purified (Qiagen) plasmid DNA of different p35PPV-NAT and pPVX full-length constructs was used for microprojectile bombardment on 4-week-old transgenic and nontransgenic *N. benthamiana* plants (four to six fully expanded leaves), using a particle inflow gun (18). Plasmid DNA was precipitated on tungsten particles (Bio-Rad) using 2.5 M $CaCl_2$ and 0.1 M spermidine. For cobombardment experiments, a mixture of two plasmids was precipitated on tungsten microprojectiles. Systemic infection was assessed visually and confirmed

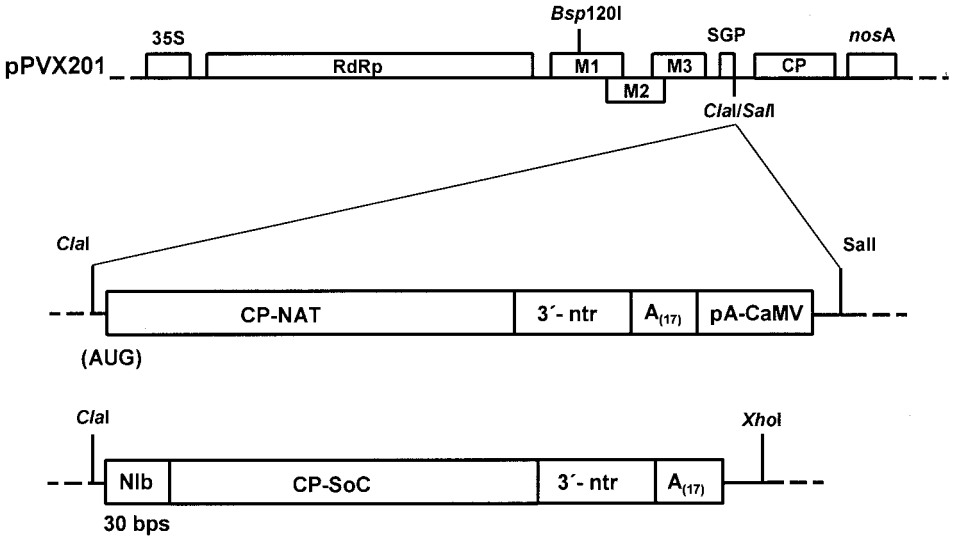

FIG. 2. PVX plant expression vector used for subcloning of CPs from different strains of PPV. M1 to M3, movement protein genes, 35S, 35S promoter of CaMV; *nos*A, nopalin synthetase gene polyadenylation signal; pA-CaMV, polyadenylation signal of CaMV; SGP, subgenomic CP promoter of PVX, $A_{(17)}$, poly(A) tail.

by plate-trapped antigen–enzyme-linked immunosorbent assay (ELISA) (23) using antiserum to the helper component protease (HC-Pro) of PPV-NAT (40).

Transgenic *N. benthamiana* expressing PPV CP. Two different transgenic lines were used for microprojectile bombardment of the different mutated p35PPV-NAT clones (Fig. 3). Transgenic homozygous *N. benthamiana* (14.27.4.) expresses a single copy of the functional CP of PPV-aphid transmissible in a tandem orientation containing the C-terminal 36 amino acid residues from the NIb protein. Only 54 bp of the PPV 3′-NTR immediately downstream of the CP gene was present in this construct. Plants of this line showed a "recovery" resistance when infected with PPV (50, 52). Plants of the homozygous transgenic line 4.30.45. transformed with a single copy of the PPV-NAT CP gene containing the complete 3′-NTR displayed the same type of resistance phenotype (25).

Cloning and sequencing of recombinant CP genes. For cloning and sequencing of the recombinant PPV CP genes, total nucleic acid (TNA) was extracted from systemically infected *N. benthamiana* plants (54) by using a modified TNA extraction buffer containing 50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.3% 2-mercaptoethanol. Primers used for amplification of a 1,324-bp CP-NAT fragment, containing the 3′-terminal part of the NIb gene and the whole CP gene, were Uni-Poty-up (5′-GGAATT CCCGCGGAAAAGCCCCGTACATTGC-3′) and Uni-Poly-T [5′-CGGGGAT CCTCGAGAAGC $(T)_{17}$-3′]. RT-PCR was carried out as described above, and PCR products were cloned into *Eco*RV-digested pBluescriptII (SK−) (Stratagene) followed by sequencing of the CP gene.

RESULTS

Different assembly mutants and CP chimeras of PPV-NAT recombine with a functional CP transgene of PPV. As described previously (52), certain CP mutations disrupted particle assembly and cell-to-cell movement of PPV. None of three assembly mutants of p35PPV-NAT were able to infect nontransgenic *N. benthamiana* plants systemically following biolistic inoculation. However, each mutant was complemented by the intact CP transgene in plant line 17.27.4., resulting in systemic infections. A similar complementation of the assembly mutants was detected in plant line 4.30.45. (Table 1). As determined visually, the previously described (52) recovery resistance in both lines was not affected by the introduced CP mutations. To test whether the accumulation of virus particles and the occurrence of systemic symptoms were due to complementation or restoration of the wild-type sequence by a recombination event, plant sap from systemically infected plants was used to inoculate nontransgenic *N. benthamiana* plants

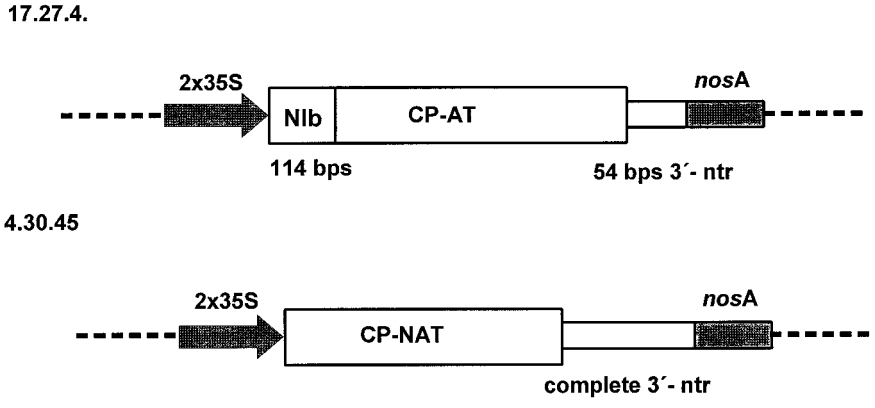

FIG. 3. CP genes of PPV in different transgenic *N. benthamiana* lines. 2×35S, enhanced CaMV 35S promoter; *nos*A, nopaline synthetase polyadenylation signal; NIb: nuclear inclusion body b.

TABLE 1. Infectivity and complementation of CP assembly mutants and CP chimeras of PPV on nontransgenic *N. benthamiana* and different transgenic plants after particle bombardment

N. benthamiana line	Infectivity and complementation[a] of:				
	-CP-RQ	-CP-DF	-CP-RQ-DF	-CP-ZYMV	-CP-PVY
Nontransgenic	− (0/15)	− (0/15)	− (0/30)	+ (8/30)	− (0/36)
17.27.4.	C (17/30)	C (3/30)	C (12/30)	+ (38/40)	C (18/26)
4.30.45.	C (5/10)	C (5/15)	C (5/10)	+ (2/10)	C (2/10)

[a] +, systemic infection; −, no systemic infection; C, complementation. The number of plants infected/number of plants inoculated is given in parentheses.

after five passages in transgenic plants (line 17.27.4.). Although the experiments were repeated three times, no systemic infection was detected, indicating that complementation but not recombination had occurred (Table 2). To confirm the retention of the different mutations after bombardment, the viral CP gene of each mutant was cloned following RT-PCR. Sequencing or restriction enzyme digests revealed the presence of the mutations in each of the three experiments. In contrast, systemic infections were observed in nontransgenic plants after one or two passages in transgenic plants containing the complete 3′-NTR adjacent to the functional CP gene (line 4.30.45.) (Table 2). The emerging CP genes were amplified by RT-PCR and cloned. The wild-type sequence of the assembly motifs was verified by restriction enzyme digests. Additional sequencing of the recombinant CP gene from PPV-CP-RQ-D revealed an exact reconstitution of a wild-type PPV sequence (Table 2). The two CP chimeric mutants of PPV-NAT (p35PPV-CP-ZYMV and p35PPV-CP-PVY) were also tested for infectivity using microprojectile bombardment. The p35PPV-NAT-CP-ZYMV chimera (Fig. 1) was able to infect *N. benthamiana* plants systemically, whereas p35PPV-PVY failed to do so (Table 1). PPV-NAT-CP-ZYMV produced milder systemic symptoms than did unmodified PPV-NAT. Systemically infected leaves were tested by ELISA using antibodies to the HC-Pro protein of PPV, leading to ELISA readings similar to those obtained in wild-type infections (data not shown). The p35PPV-NAT-CP-PVY mutant was complemented in transgenic *N. benthamiana* plants (line 17.27.4.) by the intact CP. Unexpectedly, the mutant was able to produce systemic infections in nontransgenic *N. benthamiana* plants after one passage in plant line 17.27.4. Sequencing of the core region of the CP gene revealed the originally introduced PVY CP sequence, indicating that no recombination with the intact transgenic CP transcript had occurred. In addition, 12 PVY CP clones produced in parallel were subjected to restriction enzyme digestion to test for the retention of the CP core exchange. For PPV-ZYMV chimeras, 24 clones were analyzed by restriction fragment analysis, but in no case was a recombinant sequence detected. Systemic infection of *N. benthamiana* with the PPV-PVY chimera can be explained only as second-site modifications or as mutations which might have occurred at a different part of the viral genome. Each of the two CP-chimeric mutants was maintained five times in similar passaging experiments in five 17.27.4. plants each, but the inserted ZYMV and PVY CP core regions were not exchanged with wild-type sequence by means of recombination. This was analyzed again in six (PPV-ZYMV) and 12 (PPV-PVY) different CP clones by restriction analysis. In contrast, in both CP chimeras, the wild-type PPV-NAT CP core sequence was exactly restored after one mechanical passage in plants with transgenic PPV-CP containing the complete 3′-NTR (line 4.30.45.) similar to the reconstitution of the three different assembly mutants (Table 2).

Therefore, the functional CP transgene was acquired by recombination not only if the mutant virus was unable to infect the plants systemically but also in the case of infection with viable mutants (p35PPV-NAT-CP-ZYMV and p35PPV-NAT-CP-PVY).

Recombination between assembly mutants of p35PPV-NAT and transgenic transcripts of intact PPV CP occurs on the RNA level. To demonstrate that recombination occurred during RNA replication but not between the viral cDNA clone and the transgene by a plant-encoded DNA recombinase, the assembly mutant p35PPV-NAT-CP-RQ-DF was inoculated using particle bombardment of plant line 17.27.4., in which only complementation but no recombination had been observed. An extract from systemically infected leaves containing the mutated viral RNA encapsidated by the intact transgenic CP was used for mechanical inoculation of nontransgenic plants. Mechanical inoculation of leaf extracts ensured that only virus particles but not the plasmid used for particle bombardment had been transferred. The failure to establish systemic infection provided evidence for the absence of recombination to restore the wild-type sequence. Subsequently, mechanical inoculation on transgenic plants of line 4.30.45. was carried out, and the mutant was restored to the wild-type sequence, as confirmed by the above-described restriction enzyme digest of the cloned CP gene.

Restoration of wild-type virus from assembly-defective p35PPV-NAT occurs if the intact CP is expressed by a cobombarded plant expression vector. To reproduce the recombination events obtained in transgenic plants, coinoculation of each of the three assembly mutants of p35PPV-NAT together with a PVX-based plant expression vector expressing a functional CP gene of PPV-NAT was evaluated for restoration of the wild-type PPV-NAT sequence by recombination. Since the plant virus expression vector may mask an eventually occurring PPV recombinant by systemically infecting the test plants, the PVX vector was rendered movement defective. Since the movement-defective delivery vector and the assembly-defective PPV were restricted to initially infected cells, inoculation

TABLE 2. Infectivity of CP assembly mutants and CP chimeras of PPV on nontransgenic *N. benthamiana* plants after passage in the transgenic *N. benthamiana* line 17.27.4. or 4.30.45.

Virus	Passage in 17.27.4.		Passage in 4.30.45.	
	Systemic infection	Recombination	Systemic infection	Recombination[a]
-CP-RQ	−	−	+	+
-CP-DF	−	−	+	+
-CP-RQ-DF	−	−	+	+
-CP-ZYMV	+	−	+	+
-CP-PVY	+	−	+	+

[a] All recombinants had the wild-type sequence.

TABLE 3. Cobombardment of different CP assembly mutants of p35PPV-NAT and pPVX-*Bsp*120I expressing the PPV-NAT and PPV-SoC CP genes

Clones used for cobombardment of *N. benthamiana* plants	Systemically infected plants (no. infected/ no. inoculated)
None and pPVX-CP-NAT	5/5
None and pPVX-*Bsp*120I-CP-NAT	0/5
p35PPV-CP-RQ-DF and none	0/45
p35PPV-CP-RQ and pPVX-*Bsp*120I-CP-NAT	1/40
p35PPV-CP-DF and pPVX-*Bsp*120I-CP-NAT	2/30
p35PPV-CP-RQ-DF and pPVX-*Bsp*120I-CP-NAT	6/40
p35PPV-CP-RQ-DF and pPVX-*Bsp*120I-CP-SoC	1/150

of the different clones had to be carried out using microprojectile cobombardment. This necessity has recently been shown for two defective ZYMV RNA species only after cobombardment but not after mechanical coinoculation of the different clones (16).

Bombardment of *N. benthamiana* plants with p35PPV-NAT-CP-RQ-DF or pPVX*Bsp*120I-CP-NAT did not result in systemic infections, whereas pPVX-CP-NAT caused systemic PVX infections in all of the bombarded plants (Table 3). Subsequently, 30 or 40 nontransgenic *N. benthamiana* plants were cobombarded with each of the assembly mutants of p35PPV-NAT and pPVX*Bsp*120I-CP-NAT. Two to three weeks after inoculation, one to six of the plants displayed systemic symptoms of PPV infection. The CP genes resulting from all three mutants were amplified by RT-PCR and cloned. The use of oligonucleotide Uni-Poty-up permitted amplification of the PPV CP gene but did not amplify the pPVX-expressed PPV-NAT CP gene. Restriction digestion and sequencing revealed the restoration of the wild-type sequence. The reconstitution of three different assembly mutants to the PPV wild-type sequence, resulting from transient expression of the intact CP gene, showed that as a basic principle, recombination between viral mutants and transgenic viral transcripts can be simulated in nontransgenic plants.

Recombination between assembly-defective p35PPV-NAT and pPVX-expressed CP-SoC produces a viable recombinant with chimeric CP. We investigated whether an assembly-deficient CP mutant of PPV can also be reconstituted by means of recombination with the intact CP gene of a different PPV isolate to determine possible regions of template switches of the viral replicase. For this purpose, the CP gene of PPV-SoC (36), with only 81% overall sequence similarity, was introduced into pPVX-*Bsp*120I. A total of 150 *N. benthamiana* plants were cobombarded with p35PPV-NAT-CP-RQ-DF and pPVX-*Bsp*120I-CP-SoC (Fig. 4). At 3 weeks after bombardment, one plant developed systemic PPV-like symptoms (Table 3). After TNA extraction, the amplified CP gene was cloned and sequenced. The sequence data obtained indicated that a functional CP gene was restored by a double recombination event, exchanging the core region containing the mutated assembly motifs with the corresponding part of the CP gene of PPV-SoC. Figure 5 shows the core region of the recombinant CP gene. The various nucleotide exchanges in the recombinant CP gene, derived from the CP-SoC sequence, showed that according to the template switch recombination model, the viral replicase presumably started minus-strand synthesis on the viral 3′-NTR, switched to the PVX-expressed CP transcript of PPV-SoC, and switched back to the viral genome to complete RNA synthesis.

This result shows the restoration of the defective PPV CP gene even by recombination with a related CP sequence possessing only limited sequence identity. Despite the presence of a complete 3′-NTR adjacent to the PPV-SoC CP in the PVX vector, the reconstitution was effected by a double-recombination event.

DISCUSSION

Recombination of PPV mutants in virus-resistant transgenic plants. The results obtained in our experiments corroborate the findings of previous studies in which recombination of plant viral genomes with their transgenic hosts was detected

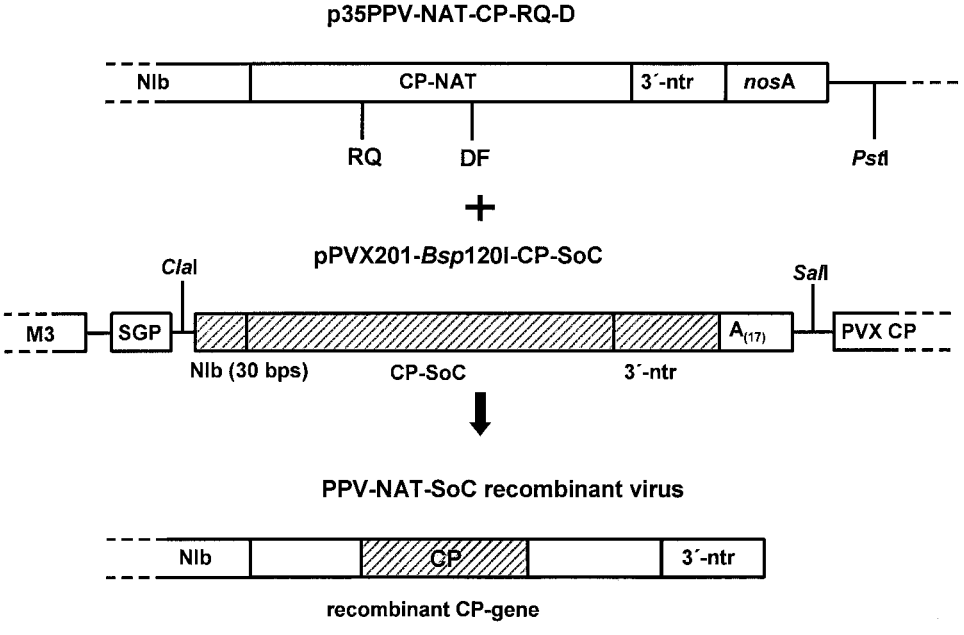

FIG. 4. Schematic representation of a chimeric PPV-NAT recombinant containing the coding sequence for the CP core of PPV-SoC, generated from p35PPV-NAT-CP-RQ-DF and pPVX-201*Bsp*120I-CP-SoC.

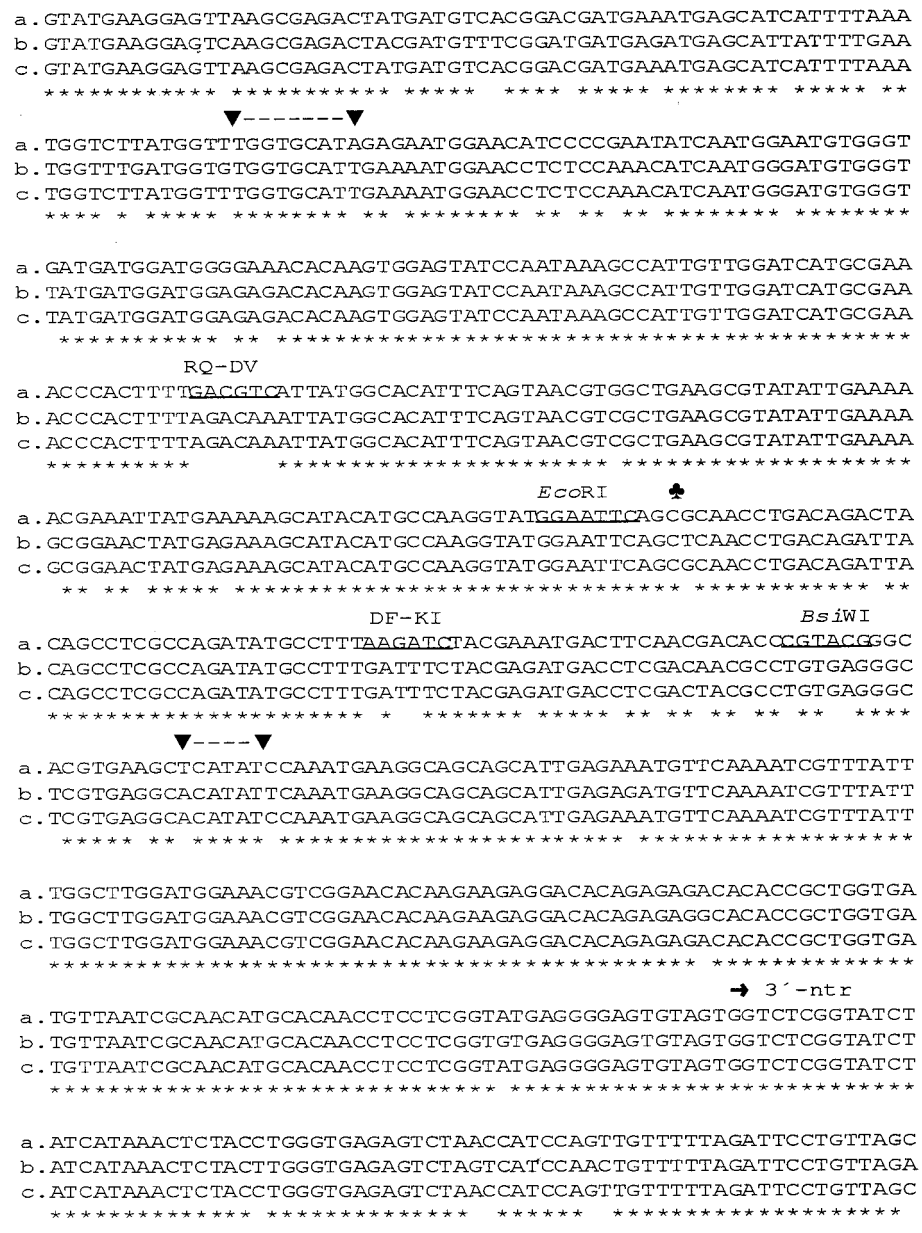

FIG. 5. ClustalX alignment (22) of the coding regions of CP-RQ-DF, CP-SoC, and the CP-NAT-SoC recombinant. *, conserved perfectly; ▼, possible region of template switch; ♣, nucleotide exchange in the recombinant sequence. (a) CP-RQ-DF (positions 8999 to 9598) (31) (GenBank accession no. D13751); (b) CP-SoC (positions 618 to 1217) (36) (EMBL accession no. X97398); (c) CP-NAT-SoC recombinant.

under experimental conditions. Our results demonstrate for the first time that potyvirus RNA in transgenic plants is available for recombination with a challenging potyvirus mutant.

In a widely accepted model, two types of RNA recombination have been defined (26). Based on the similarity of the parental RNA molecules, there are homologous (HR) and nonhomologous (NHR) recombination types, the former of which is divided into precise and imprecise recombination. All PPV mutants or CP chimeras in our experiments were restored exactly to the wild-type sequence, revealing a precise HR type. This is comparable to *Tomato bushy stunt virus* (TBSV) recombinants, which were also identical to wild-type virus (4), but in contrast to an imprecise HR type in recombinant CCMV in transgenic plants, which had several modifications at putative crossover sites (19).

The recombination experiments shown here were designed for application of different selection conditions for the occurrence of a viral recombinant. The three assembly mutants of p35PPV-NAT were capable of systemically infecting the plants only when they had recombined with the intact homologous transgene. The selection pressure was conferred by essential particle assembly and cell-to-cell movement. The CP chimera p35PPV-NAT-CP-ZYMV, however, recombined to the wild-type sequence, although the mutant was still able to form particles and to spread in *N. benthamiana* plants systemically. Therefore, the recombined wild-type virus must have had a

selective advantage over the chimera, since it was no longer detected after passage through nontransgenic *N. benthamiana* plants. We conclude that the selection pressure is moderate in this case.

The p35PPV-NAT-CP-PVY chimera acquired the ability to infect nontransgenic plants following adaptation during replication in plants of line 17.27.4. This could have taken place by second-site modification somewhere in the genome. The restoration of the wild-type sequence after one passage in plants of line 4.30.45. can be assigned to the condition of moderate selection pressure, similar to the ZYMV chimera. By analogy to recombination in CaMV (56) and TBSV (4), recombination of a PPV CP mutant with the ability to induce systemic infections was demonstrated. It has been previously suggested that viral recombination in transgenic plants can be detected only if the mutant gains a significant advantage from this event (11, 46). It seems to be difficult to define a certain threshold of selection pressure or selective advantage which is necessary for the mutant virus to acquire the transgene. Furthermore, nothing is known about a possible competition between the chimera and the wild-type virus in transgenic plants. Therefore, it can only be assumed that in the case of the two CP chimeras of PPV, a slight selective advantage combined with an absolute sequence homology of the adjacent sequence must have been sufficient to ensure the relatively rapid restoration of the wild-type virus.

In contrast, our results showed that the transgenic sequence must also meet certain requirements to enable restoration to occur by recombination, even if high selection pressure was applied. If the region necessary for replication initiation was shortened in the transgenic sequence, the assembly mutants and the CP chimeras were maintained and remained unchanged over months in several passages through transgenic plants. This was presumably due to complementation without the need to restore a wild-type sequence, thereby strengthening the proposed recombination model (20). If a double-template switch of the viral replicase is necessary for restoration, it was assumed that a minimal transcript length must be available for two template switches. An example of restoration of an intact virus by a double-template switch was provided for two ZYMV mutants containing deletions in different parts of the genome (16). However, the possible regions for two template switches were several times longer than the transgenic CP sequence used in our experiments. Collectively, this demonstrated that recombination occurs under high and moderate selection pressure and that the transgenic 3′-NTR may be utilized by the viral replicase complex for replication initiation, thereby enhancing the probability of recombination and enabling the reconstitution of viable virus with a single template switch.

It should be stressed that the two different plant lines used in the recombination experiments showed a recovery resistance to PPV (i.e., plants recovered from PPV infections). This allowed the infecting mutants to replicate and to spread with the aid of the transgenic CP before viral RNA was degraded. In none of the recombination experiments so far reported for transgenic plants did the transgenic plants confer effective virus resistance when inoculated with the wild-type virus. When comparing recovery resistance with immunity or extreme resistance, in which most of the viral RNA is degraded before plants become systemically infected, it can be assumed that the number of possible recombinations is much smaller due to the lower replication rates. Thus, the use of the recovery-resistant plants in our experiments showing the complementation phenomenon may have enhanced the possibility of recombination.

How can our results be used to define the recommendations for the elimination of the possible recombination risks when exploiting the virus resistance conferred by transgenic plants (2)? In addition to the removal of replication initiation sites and the shortening of the viral transgene to minimize the recombination target, known functions can be disrupted by mutating the corresponding coding regions. Virus resistance in transgenic plants has been established by using shortened viral genes as small as about 400 bp (37). On the other hand, we have previously shown that the assembly-defective PPV genes elicit levels of resistance similar to that elicited by the wild-type gene (52). Finally, only plants with high levels of resistance should be selected from transformation experiments so as to reduce the amount of replicating viral RNAs.

Recombination between CP-defective PPV and transiently expressed CP genes of different PPV strains. The attempt to reproduce the restoration of assembly-defective PPV in transgenic plants carrying an intact CP gene transiently expressed from a PVX-based plant expression vector yielded positive results. This is the first example of recombination between a defective plant virus and corresponding transcripts produced by a different virus. This shows that the PPV replicase complex not only switches to transgenic transcripts to restore CP defects by precise HR but also switches to viral transcripts of PVX replicating in the same cell. Apparently, this offers the possibility of screening viral genome parts of different sizes or different origins for their availability for homologous recombination. This may provide an opportunity to determine the critical length for recombination and to define possibly preferred crossover sites of the viral replicase complex. In an additional experiment, we replaced the PPV-NAT CP gene in the PVX genome with the PPV-SoC CP gene and thus were able to restore the CP assembly mutant of PPV, generating a functional chimeric CP gene by means of precise HR. The resulting sequence (Fig. 5) enabled us to identify the putative recombination junction sites in short segments of sequence identity. Another recovery of chimeric viruses under experimental conditions has previously been reported (33).

Based on the findings with transgenic plants where the intact 3′-NTR contributed to recombination, a similar result would have been expected for PVX-mediated expression of the intact PPV-SoC CP gene. However, in accordance with the RdRp template switch recombination model, restoration was achieved by a double-template switch of the replicase despite the existence of a complete 3′-NTR of the PPV-SoC for replication initiation expressed by PVX. The possibility that the 3′-NTR of PPV-SoC, with about 95% sequence identity to that of PPV-NAT, does not allow replication initiation seems unlikely. The 3′ adjacent PVX genome might have inhibited the formation of RNA secondary structures, which are required for efficient initiation (21). Finally, this may also indicate that there are fewer constraints on recombination among viral RNAs than between viral RNAs and viral transgenes. This hypothesis must remain unanswered until this in vivo recombination system has yielded more experimental data. In addition, it is possible that the very low frequency of recombination can be explained by the limited sequence identity or by the necessity for an unlikely double-template switch. However, this system seems useful for the production of viral recombinants in vivo and for testing of the minimal sequence identity required for recombination.

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