Development of an antigen presentation system based on plum pox potyvirus

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Abstract The development of an antigen presentation system based on the plum pox potyvirus (PPV) is here described. The amino-terminal part of PPV capsid protein was chosen as the site for expression of foreign antigenic peptides. Modifications in this site were engineered to avoid the capability of natural transmission by aphids of this PPV vector. As a first practical attempt, different forms of an antigenic peptide (single and tandem repetition) from the VP2 capsid protein of canine parvovirus (CPV) were expressed. Both chimeras are able to infect *Nicotiana clevelandii* plants with similar characteristics to wild-type virus and remain genetically stable after several plant passages. The antigenicity of purified chimeric virions was demonstrated, proving the suitability of this system for diagnostic purposes. Moreover, mice and rabbits immunized with chimeric virions developed CPV-specific antibodies, which showed neutralizing activity.

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Key words: Virus vector; Plant expression system; Potyvirus; Vaccine; Parvovirus

1. Introduction

Nowadays, plants are being engineered as novel biomanufacturing systems. Several advantages are offered by plants in comparison to other expression systems. In particular, they are an inexpensive, safe and easy way to obtain proteins of interest, not needing expensive manufacturing facilities for the scaling-up, and allowing the replacement of traditional surplus crops. The production of proteins of potential pharmaceutical value or subunit vaccines in plants is of particular interest [1].

There are two major strategies for plant production of therapeutic molecules: genetic transformation of the nuclear genome of plants to create transgenic plants, and manipulation of the genome of plant viruses [1]. The latter strategy has the advantage of causing plants to produce relatively large amounts of the desired protein at a determined moment of their developmental cycle; moreover, virus stocks can be maintained without passages through plants for long periods of time. In the particular case of peptide vaccines, the immunogenicity of chemically synthesized peptides is enhanced by conjugation to larger carrier molecules, especially when these carrier molecules have a particulate structure [2]. The linkage of epitopes on the surface of plant viral particles is, therefore, a potential way to improve antigen presentation and to obtain an effective antibody response. Both filamentous and icosahedral plant viruses have been successfully developed as epitope presentation systems [2–8] and in some cases as an alternative to previous tissue culture-derived vaccines [9].

Plum pox virus (PPV) is a member of the plant potyvirus group. The helicoidal virions are comprised of a messenger polarity RNA molecule surrounded by more than 2000 copies of a single capsid protein (CP) [10]. The potyvirus CP has been implicated in several functions during the virus life cycle; apart from the obvious function in encapsidation, this protein holds an amino acid sequence (triad Asp-Ala-Gly) that is essential for aphid transmission [11,12]. CP has also been involved in potyvirus movement inside the plant for both cell-to-cell and systemic movement. In particular, the amino- and carboxy-terminal parts of CP have been implicated in long distance movement [13–15]. Besides, both the amino- and carboxy-terminal parts of the CP have been previously described to be surface exposed on the potyvirus virions and to be highly immunogenic [16,17]. Naturally appearing PPV mutants called NAT (non-aphid-transmissible) have been reported [18,19]. These mutants have a 15 amino acid (aa) deletion in the amino-terminal part of CP; this deletion includes the glycine of the amino acid triad essential for transmission by aphids.

The VP2 protein is the major structural protein of the canine parvovirus (CPV). There have been extensive studies and fine mapping of epitopes of the VP2 protein amino-terminal part, and synthetic vaccines against CPV based on peptides have been described [20]. Among others, the 6L15 peptide has previously shown the ability to induce neutralizing antibodies against CPV in both mice and rabbits [21].

In this paper, the construction of an antigen presentation system based on PPV is described. A full-length cDNA clone of PPV [22] was used to engineer a mutant with a NAT-like deletion and a unique cloning site *Mlu*I, which enables us to introduce foreign sequences in that specific region. This system was used to produce chimeric PPV CPs containing immunologically active CPV-neutralizing epitopes.

2. Materials and methods

2.1. Construction of the chimeric clones

The *Bam*HI-*Sac*I fragment that contains the sequence encoding the amino-terminal part of CP (extracted from the PPV full-length cDNA clone pGPPV [22]) was introduced in M13mp19. Site-directed mutagenesis was performed using the oligonucleotides 5′-GTGGTT-GAAGTGCATCAACTTC-3′ and 5′-GAGGTGGTTGTAACGCG-TCAACTTCC-3′ to create the NAT and NATMluI mutations, respectively. The mutagenesis products were verified by sequencing, and their *Bam*HI-*Sac*I fragments were cloned back in the full-length cDNA clone creating pGPPV-NAT and pGPPV-NATMluI.

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An intermediate clone that contains the *Bam*HI-*Sac*I fragment of pGPPV-NATMluI inserted in pUC18 (pUC18MluI) was constructed to facilitate the cloning of sequences encoding antigenic peptides. The sequence encoding the 6L15 CPV peptide was created by hybridizing complementary oligonucleotides (plus strand: 5′-CGCGGTTCAAC-CAGACGGTGGTCAACCTGCTGTCAGAAATGAACG-3′; minus strand: 5′-CGCGCGTTCATTTCTGACAGCAGGTTGACCACCG-TCTGGTTGAAC-3′) which produced *Mlu*I-compatible overhangs. The hybridization was carried out by incubating 1.5 μg of each oligonucleotide in a buffer containing 10 mM Tris-HCl pH 7.5, 100 mM NaCl and 1 mM EDTA at 90°C for 5 min, and then cooling down slowly to room temperature. The hybridization mixture was ligated with *Mlu*I-digested pUC18MluI, creating pUC18CPV. The *Bam*HI-*Sac*I fragment of this plasmid, which contains the sequence encoding the 6L15 peptide, was then introduced in the PPV full-length cDNA clone, creating pGPPV-CPV.

To make the dimer-epitope clone pGPPV-2CPV, we followed the same procedure described above, but the oligonucleotides were phosphorylated with T4-polynucleotide kinase before hybridization, and pUC18MluI was treated with alkaline phosphatase after *Mlu*I digestion. This allowed us to obtain pUC18-derived clones containing more than a copy of the sequence coding for the CPV peptide. Clones with two copies in the correct orientation were selected. The same procedure as explained for one copy clone was performed to construct pGPPV-2CPV from the intermediate clone.

2.2. In vitro transcription and plant inoculation

Capped transcripts from full-length cDNA clones were synthesized with the T7 Cap Scribe transcription kit (Boehringer Mannheim), as per the manufacturer's instructions. 20 μl of reaction mixture diluted 1:1 in 5 mM sodium phosphate buffer pH 7.5 was used to inoculate eight plants dusted with carborundum (three leaves per plant).

2.3. Western blot analysis

Samples from infected plants homogenized in PBS-Tween-PVP buffer (1×PBS, 0.5 g/l Tween 20 and 2% polyvinylpyrrolidone K25) [23] or in 5 mM sodium phosphate pH 7.5 were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and subjected to immunoreaction as described [24]. The anti-PPV monoclonal antibody (MAb) 5B was obtained from REALISA kit (Durviz). The anti-CPV MAb 3C9 has been previously described [25]. The second antibody was a peroxidase-conjugated goat anti-mouse IgG purchased from Jackson ImmunoResearch Laboratories. The peroxidase reaction was developed with 4-chloro-1-naphthol.

2.4. Immunocapture RT-PCR (IC-PCR)

Samples from infected plants homogenized in PBS-Tween-PVP buffer were incubated for 2 h at 37°C in tubes previously coated with anti-PPV IgG, and then, after two washing steps with PBS-Tween buffer, reverse transcription-PCR (RT-PCR) was performed as previously described [23].

2.5. Immunization of animals

Groups of five 3-week-old BALB/c mice were immunized intraperitoneally on days 0 and 20. Different amounts of purified PPV-CPV, PPV-2CPV and PPV virions in PBS were emulsified in the same volume of complete Freund's adjuvant (Sigma) for the first inoculation and incomplete Freund's adjuvant (Sigma) for the subsequent booster. Immunized mice were bled on days 0 and 35, and the sera were pooled and tested by ELISA for titers of anti-peptide, anti-CPV and anti-PPV antibodies, and by a monolayer protection assay for CPV-neutralizing activity.

For New Zealand White rabbits (5–6 weeks old), the antigen used for immunization varied between 5 μg and 1 mg of chimeric PPV-CPV or PPV-2CPV as indicated in the tables. Groups of two or three animals received two injections with the same dose of chimeric virus with a 21-day interval, by the intramuscular route, with complete and incomplete Freund's adjuvants (Sigma), respectively. Blood samples were taken before starting experiments and 15 days after the second immunization, and sera were tested as before.

2.6. ELISAs

For antigenicity assays, a DASI-ELISA was carried out with increasing amounts of PPV, PPV-CPV and PPV-2CPV purified virions following the protocol described in the REALISA kit (Durviz). The anti-PPV MAb 5B (supplied in the kit) and the anti-CPV MAb 3C9 were used in the assays.

Three indirect ELISAs were carried out to detect peptide-, CPV- and PPV-specific antibodies in immunized animals. Peptide 2L21 which corresponds to the N-terminus of CPV VP2 [21] was synthesized in *Escherichia coli* as a ubiquitin-fusion protein (kindly provided by Igen, USA). Peptide 2L21, purified CPV and purified PPV-R3, respectively, were used as antigen sources. Polystyrene microtiter plates (Labsystem, Finland) were coated with the peptide, purified CPV or PPV at 5 mg/ml diluted in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. Plates were incubated at 37°C for 1 h with the first antibody (rabbit or mouse sera) diluted in blocking buffer (350 mM NaCl, 0.05% Tween 20 in PBS). Peroxidase-labeled protein A (Sigma) or anti-mouse IgG (Pierce) were diluted 1/1000 in blocking buffer and used as corresponding conjugate by incubation for 1 h at 37°C. Washes were performed with PBS containing 0.05% Tween 20. Horseradish peroxidase activity was detected by adding ABTS (2,2′-azino-di(ethyl-benzothiazoline)) (Sigma) as substrate and stopped after 15 min with 2% SDS. The optical density of samples was determined at 405 nm (Bio-Tek Instruments).

2.7. In vitro protection assays

To determine the ability of the specific rabbit and mouse sera to neutralize the virus in vitro, a CRFK cell monolayer-protection assay was performed as previously described [25].

2.8. Immunogold labeling assay

10 μl of a 0.1 μg/ml dilution of PPV and PPV-CPV were adsorbed onto formvar grids for 2 min. After an 8 min washing step with TBG buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% gelatine, 0.1% bovine serum albumin) both samples were incubated with either a polyclonal antibody against PPV or MAb 3C9 for 8 min. After five washing steps with TBG buffer, samples were incubated for 8 min with 5 nm gold-labeled anti-rabbit IgG or 10 nm gold-labeled anti-mouse IgG, depending on the first antibody used. Grids were finally washed five times with TBG buffer and three times with milliQ water before staining with 2% uranyl acetate for 30 s. Micrographs were obtained in a JEOL 1200EXII electron microscope.

3. Results

3.1. Construction of an expression vector based on PPV

As a first attempt to express foreign sequences in the amino-terminal part of PPV CP, we decided to replace PPV sequences by foreign ones. NAT mutants are natural precedents of viable PPV CP deletion mutants. NAT-imitating mutants were created by site-directed mutagenesis of the PPV full-length clone pGPPV [22]. Two mutated full-length clones were constructed. pGPPV-NAT has the 45 nt deletion found in natural NAT mutants, but no other sequence changes compared to pGPPV. pGPPV-NATMluI has the same deletion as pGPPV-NAT but also additional nucleotide changes near the deletion point, which create a unique *Mlu*I restriction site (Fig. 1A). This new construct allowed us to clone foreign sequences encoding the peptides of interest.

Capped transcripts from the mutant and wild-type clones were inoculated onto *Nicotiana clevelandii* plants. Both mutants were able to infect plants. The time course of infection and symptomatology were similar to those of wild-type-infected plants. Western blot analysis of infected plants showed that PPV-NAT and PPV-NATMluI mutants accumulated to a similar extent as wild-type PPV. As expected, the CPs of the mutant viruses showed higher electrophoretic mobilities than wild-type CP (Fig. 2A). IC-PCR of samples from infected plants amplified bands of the expected sizes for each mutant (Fig. 2B). The stability of the mutated sequence in the progeny virus was corroborated by *Mlu*I digestion (Fig. 2B) and nucleotide sequencing of the IC-PCR-amplified products (data not shown).
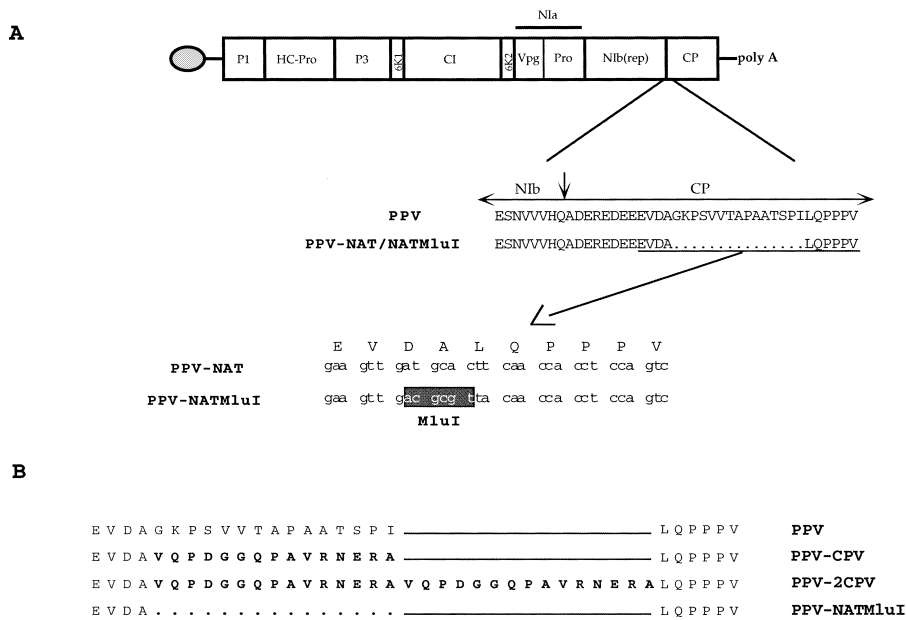
Fig. 1. Schematic representation of the mutant and chimeric PPV constructs. A: NIb and CP amino acid sequences of PPV, PPV-NAT and PPV-NATMluI at the deletion site are compared, indicating their position in the genomic map of the virus. Deleted amino acids are represented by points. CP cistron nucleotide sequences of PPV-NAT and PPV-NATMluI are also compared at that site. The black box shows the *Mlu*I restriction site. B: Amino acid sequence of PPV, PPV-CPV, PPV-2CPV and PPV-NATMluI at the NAT deletion site. Foreign and deleted amino acids are represented by bold letters and points, respectively.

3.2. Construction and purification of PPV-CPV chimeras

The pGPPV-NATMluI clone was used to introduce foreign sequences in the PPV genome. A 45 nt CPV sequence that reestablished the number of amino acids of wild-type PPV-CP was introduced at the *Mlu*I site creating pGPPV-CPV (Fig. 1B). This sequence encodes a 15 aa peptide of CPV VP2 protein that had previously been described to induce complete protection when experimental animals were immunized with it [20]. To test if this site could accommodate larger sequences and if a tandem repetition of the CPV epitope could improve the immunogenicity of the chimera, a dimer of the CPV sequence was introduced, creating pGPPV-2CPV. This clone contains a 90 nt foreign sequence and a 45 nt increased size compared to PPV wild-type genome, which results in a CP 15 aa larger than wild-type (Fig.

1B). Transcripts from both chimeras were able to infect *N. clevelandii* plants with similar characteristics to those of wild-type.

Virions were purified from *N. clevelandii* plants infected with PPV, PPV-CPV and PPV-2CPV. The yield after purification of both chimeric viruses was similar to that of PPV and there seemed to be no difference in virion stability either after purification treatments or under commonly used storage conditions.

3.3. Immunocharacterization of chimeric viruses

Purified virion samples were analyzed by Coomassie blue staining and by immunoreaction against PPV- and CPV-specific MAbs. Although PPV and PPV-CPV CPs have the same number of amino acids, they have a slightly different electro-
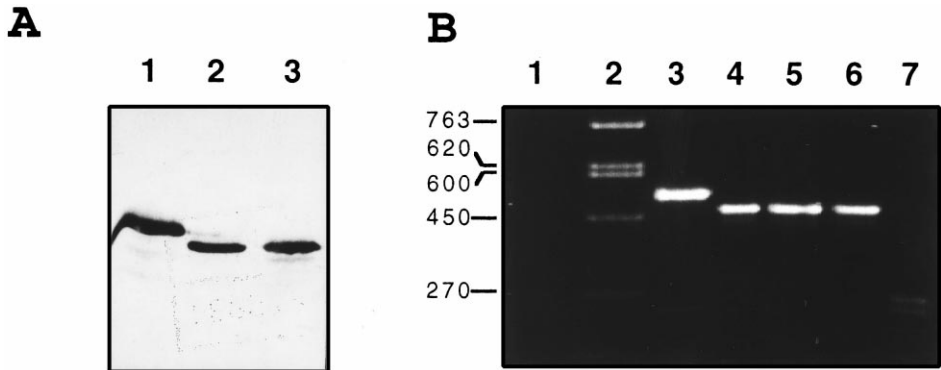

Fig. 2. Characterization of mutant PPV accumulated in systemically infected *N. clevelandii* leaves. A: Western blot analysis of extracts from plants infected with transcripts synthesized in vitro from pGPPV (lane 1), pGPPV-NAT (lane 2), and pGPPV-NATMluI (lane 3). The immunoreaction was carried out with the anti-PPV MAb 5B. B: IC-PCR products (nt 8390–8900 of the PPV genome [28]) amplified from a healthy plant (lane 1) and from plants infected with wild-type PPV (lane 3), PPV-NAT (lane 4) and PPV-NATMluI (lane 5). Lanes 6 and 7 show the result of *Mlu*I digestion of the PPV-NAT and PPV-NATMluI amplification products, respectively. *Hin*dIII restriction fragments of phage ϕ29 DNA were used as size markers (lane 2).

A

1   2   3   4   5   6   7   8   9

B


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763
620
600
450

270

170
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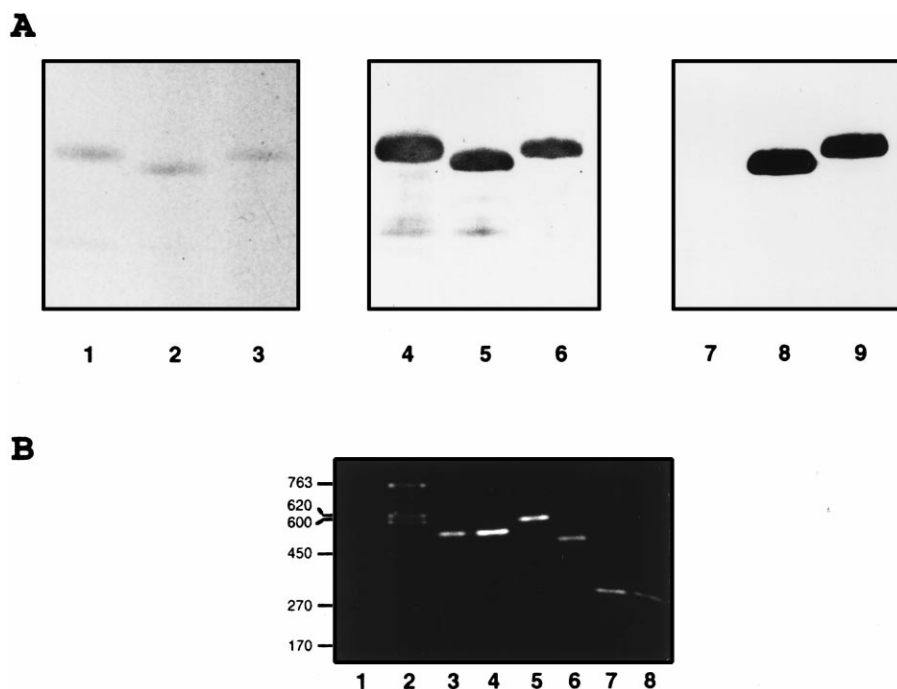
1 2 3 4 5 6 7 8

Fig. 3. Characterization of chimeric PPV. A: Coomassie blue staining (lanes 1–3), Western blot analysis with anti-PPV MAb 5B (lanes 4–6) and anti-CPV MAb 3C9 (lanes 7–9) of wild-type PPV (lanes 1, 4 and 7), PPV-CPV (lanes 2, 5 and 8) and PPV-2CPV (lanes 3, 6 and 9) purified virions. B: IC-PCR products (nt 8390–8900 of the PPV genome [28]) amplified from a negative control (lane 1) and from wild-type PPV (lane 3), PPV-CPV (lane 4) and PPV-2CPV (lane 5) purified virion samples. Lanes 6, 7 and 8 show the *Hinc*II digestion of wild-type PPV, PPV-CPV and PPV-2CPV amplification products, respectively. *Hind*III restriction fragments of phage φ29 DNA were used as size markers (lane 2).

phoretic mobility, as expected from their different amino acid composition (Fig. 3A). The DNA fragments amplified by IC-PCR corresponding to the CP amino-terminal region of PPV and PPV-CPV showed the same size (Fig. 3B), indicating that no deletions were taking place in the chimeric virus inserted sequence. The DNA fragment amplified from the PPV-2CPV chimera showed the expected size increase compared to PPV and PPV-CPV. These conclusions were confirmed by restriction digestion with *Hinc*II as there is a restriction site in the CPV sequence (Fig. 3B), and by sequencing of the amplified products, which also verified the absence of second mutations introduced during replication of the chimeric viruses (data not shown). Some chimeras based on plant viruses encoding foreign sequences have shown a tendency to lose the foreign insert after a number of replication rounds or plant passages [6]. PPV-CPV and PPV-2CPV chimeric viruses were genetically stable in plants after up to 30 days and PPV-CPV mutant remained stable after four subsequent passages in *N. clevelandii* plants (data not shown).

As expected, all purified virions were recognized by MAb 5B raised against PPV CP in Western blot analysis. Only PPV-CPV and PPV-2CPV virions were recognized by MAb 3C9 against the CPV epitope. There was a strong reaction of the latter antibody with both chimeras and no background reaction with wild-type PPV (Fig. 3A).

The antigenicity of the chimeric viruses was assayed by DASI-ELISA (Fig. 4). All viruses were recognized at similar levels by the anti-PPV antibody, the saturation being reached above 100 ng. As expected, wild-type PPV was not recognized by the 3C9 antibody. PPV-CPV and PPV-2CPV chimeras reacted with this anti-CPV MAb. ELISA values for PPV-2CPV were somewhat higher, reaching saturation at lower amounts

than PPV-CPV, probably reflecting the fact that PPV-2CPV holds two copies of the 6L15 epitope.

Immunogold labeling experiments were performed comparing PPV and PPV-CPV. The morphology of both virus particles seemed to be similar. Both PPV and PPV-CPV were gold-labeled when incubated with a polyclonal antibody against PPV, but only PPV-CPV was labeled when the incubation was instead performed with MAb 3C9 (Fig. 5).

3.4. Immunogenicity of the PPV-CPV chimeras

To test the ability to stimulate production of CPV-specific antibodies, several amounts of PPV-CPV chimeras were used to immunize mice. Table 1 shows ELISA titrations at day 35
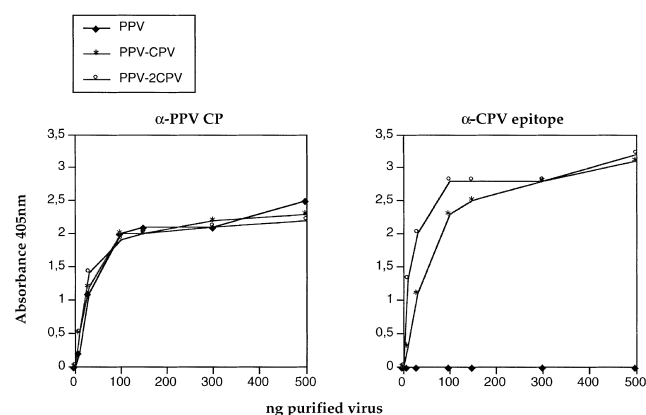

Fig. 4. DASI-ELISA of PPV, PPV-CPV and PPV-2CPV purified virions using anti-PPV MAb 5B (left) and anti-CPV MAb 3C9 (right).
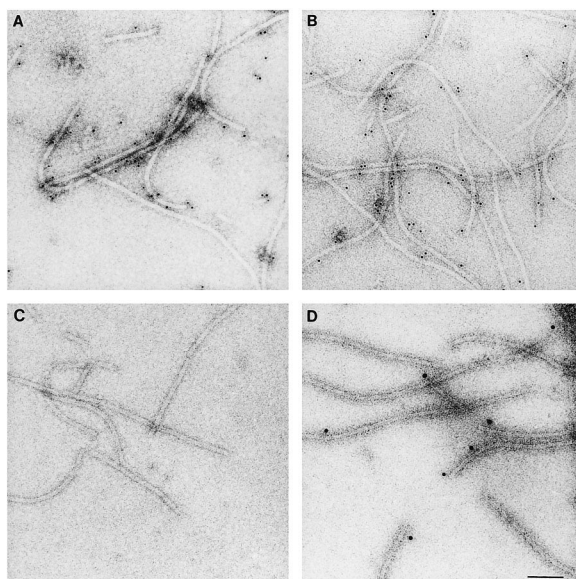
Fig. 5. Immunogold labeling of PPV (A and C) and PPV-CPV (B and D) incubated with an anti-PPV polyclonal antibody (A and B) and anti-CPV MAb 3C9 (C and D).

of the various sera expressed as log reciprocal antibody dilution. All mice developed antibodies to PPV showing a good correlation between the amount of antigen used and anti-PPV titer. Doses as low as 0.05 μg of PPV-2CPV were able to induce anti-peptide and anti-CPV antibodies, albeit at low titers, 1.7 and 1.0, respectively. The strongest response against peptide and CPV was developed in mice immunized with 5 μg of PPV-2CPV (group 6), reaching ELISA titers of 4.4 and 3.7 for anti-peptide and anti-CPV antibodies, respectively. These titers were slightly higher than those obtained in mice immunized with the same amount of chimeric particles containing the monomer (group 2). Group 3, immunized with the highest dose (50 μg), responded to CPV peptide to a significantly lower extent.

To study the immunogenicity of the chimeric particles in rabbits, a second immunization experiment was carried out. Seven groups of two or three rabbits were immunized with different doses of PPV-CPV or PPV-2CPV with a 21-day interval (Table 2). In general, anti-PPV antibody levels increased with the antigen dose. All rabbits immunized with chimeric particles elicited detectable anti-peptide and anti-CPV responses. The highest antibody titers were obtained when rabbits were immunized with 100 μg/dose of PPV-CPV, reaching titers of 4.1 for the peptide and 3.7 for CPV. The second highest antibody titers were observed in rabbits immunized with 500 μg/dose of PPV-CPV or PPV-2CPV, suggesting that the most effective amount of chimeric particles to stimulate production of CPV-specific antibodies in rabbits was between 100 and 500 μg, administered in two doses. The use of higher doses like 1 mg did not increase proportionally the CPV-specific response as observed in mice, probably due to antigenic saturation. The insertion of dimers instead of monomers of the epitope sequence seems not to increase significantly the rabbit CPV-specific response in contrast to that observed in mice.

3.5. CPV-neutralizing antibody response elicited by the PPV-CPV chimeras

To assess the ability of the chimeric PPV-CPV particles to induce CPV-neutralizing antibodies, a monolayer-protection assay was carried out with immunized mouse and rabbit sera in the absence of complement. Tables 1 and 2 show the neutralization titers of mouse and rabbit sera, respectively. All mice elicited detectable levels of CPV-neutralizing antibodies, except group 4, immunized with 0.05 μg of PPV-2CPV, which showed almost no response to CPV or PPV. The highest neutralizing response was achieved by group 6 mice immunized with 5 μg of PPV-2CPV, with a titer of 3.2, which is slightly higher than the 2.6 titer obtained with the same dose of monomer chimeric particles.

In the rabbit immunization experiment, 12 out of 16 animals showed detectable CPV-neutralizing antibodies, ranging between titers of 1.0 for rabbits immunized with 5 μg of PPV-CPV and 2.9 for rabbits immunized with 100 μg of PPV-CPV or 500 μg of PPV-2CPV, confirming that the most adequate amount of chimeric particles for rabbits was between 100 and 500 μg. Sera from rabbits immunized with chimeric particles containing the dimer were not especially effective in inducing

Table 1
Antibody response to chimeric PPV-CPV and PPV-2CPV particles in mice

Mouse group	Dose	ELISA			NT[a]
		Peptide[b]	CPV[c]	PPV[d]	
1	0.5 μg PPV-CPV	3.8[e]	2.8	4.4	2.3[f]
2	5 μg PPV-CPV	4.1	3.4	5.0	2.6
3	50 μg PPV-CPV	2.6	1.9	5.3	1.9
4	0.05 μg PPV-2CPV	1.7	1.0	2.3	–
5	0.5 μg PPV-2CPV	3.8	2.5	4.4	1.9
6	5 μg PPV-2CPV	4.4	3.7	5.3	3.2
7	5 μg PPV	–	–	5.0	–
8	50 μg PPV	–	–	5.0	–

[a]CPV neutralization assay as described in Section 2.
[b]ELISA for anti-peptide antibodies. Peptide 2L21 was used as antigen.
[c]ELISA for anti-CPV antibodies. Purified CPV was used as antigen.
[d]ELISA for anti-PPV antibodies. Purified PPV was used as antigen.
[e]ELISA titers were determined 2 weeks after the second immunization and are expressed as −log of serum dilution which yielded absorption values three times the blank (preimmunization serum). Average values for all group mice are shown. –: no detectable immunoreaction (−log of lowest serum dilution tested: 1.0).
[f]Neutralization titers were determined 2 weeks after the second immunization and are expressed as −log of highest serum dilution able to protect cell monolayer from CPV infection. –: no detectable neutralizing activity (−log of lowest serum dilution tested: 1.0). Average values for all group mice are shown.

Table 2
Antibody response to chimeric PPV-CPV and PPV-2CPV particles in rabbits

Rabbit group	Dose	ELISA			NT[a]
		Peptide[b]	CPV[c]	PPV[d]	
1	5 µg PPV-CPV	2.0 ± 0.3[e]	1.6 ± 0.6	3.8 ± 0.0	0.5 ± 0.5[f]
2	50 µg PPV-CPV	2.4 ± 0.5	1.4 ± 0.8	4.4 ± 0.0	1.4 ± 0.9
3	100 µg PPV-CPV	3.5 ± 0.5	3.0 ± 0.6	4.3 ± 0.2	2.3 ± 0.6
4	500 µg PPV-CPV	3.5 ± 0.0	2.8 ± 0.0	4.7 ± 0.0	2.4 ± 0.2
5	500 µg PPV-2CPV	3.5 ± 0.0	2.8 ± 0.0	4.5 ± 0.2	2.6 ± 0.3
6	1 mg PPV-CPV	2.5 ± 0.6	1.0 ± 0.6	4.5 ± 0.3	1.0 ± 0.7
7	50 µg PPV	–	–	4.4 ± 0.0	–
8	500 µg PPV	–	–	4.4 ± 0.0	–

[a]CPV neutralization assay as described in Section 2.
[b]ELISA for peptide-specific antibodies. Peptide 2L21 was used as antigen.
[c]ELISA for CPV-specific antibodies. Purified CPV was used as antigen.
[d]ELISA for PPV-specific antibodies. Purified PPV was used as antigen.
[e]ELISA titers were determined 2 weeks after the second immunization and are expressed as −log of serum dilution which yielded absorption values three times the blank (preimmunization serum). Data represent the means of values ± S.D. for every rabbit group. –: no detectable immunoreaction (−log of the lowest serum dilution tested: 1.0).
[f]Neutralization titers were determined 2 weeks after the second immunization and are expressed as −log of highest serum dilution able to protect cell monolayer from CPV infection. –: no detectable neutralizing activity (−log of the lowest serum dilution tested: 1.0).

neutralizing activity, which is in agreement with the ELISA results. As expected, rabbit sera with a very low response against VP2 peptide were not capable of neutralizing CPV.

4. Discussion

Viruses have been demonstrated to be useful expression vectors in plants [26,27]. In particular, different plant viruses have been developed as presentation systems for the expression of foreign peptide sequences [2–8]. The PPV-based vector reported in this paper is, to our knowledge, the first description of a potyvirus being used for this purpose.

The amino-terminal part of the virus CP was chosen as cloning site for two reasons. First, it is encoded by the most variable region, in length and sequence, of the potyvirus genome. This fact, suggesting a potential capability of the potyvirus genome to hold different sequences in length in this region, is especially remarkable taking into account that some viral systems only tolerate short genome insertions. Second, the amino-terminal part of the PPV CP is exposed on the virion surface and is highly immunogenic [17].

NAT mutants are natural examples of modifications in the amino-terminal part of PPV CP. A PPV-based expression vector (PPV-NATMluI) was constructed by removing from a full-length cDNA clone the dispensable region deleted in these natural mutants, and introducing a restriction site to facilitate the cloning of foreign sequences. This vector should be restricted to inoculated plants since part of the CP region required for aphid transmission is included in the deleted sequence. This safety point is very relevant with reference to large-scale production in the field.

A CPV antigenic peptide sequence, in both single and tandem form, was cloned in the PPV-NATMluI vector. The two chimeras (PPV-CPV and PPV-2CPV) were viable and genetically stable, showing that the insertion site is somehow permissible to modification and that inserts as long as 30 amino acids can be tolerated without any deleterious effect on virus growth. The amino-terminal part of potyviruses has been implicated in systemic movement [13,14], the mutants described here seem to have no reduced long distance movement, so sequences required for such function have to be restricted to other parts of the CP amino-terminal region. The yield of the

chimeric virus particles is similar to that of wild-type PPV, besides chimeric virions are stable after purification treatments and storage.

The particulate and repetitive structure of potyvirus virions (about 2000 copies of a single protein), together with the surface exposure of the potyvirus CP amino-terminal part, presumes that chimeras based on PPV-NATMluI might behave as competent antigen delivery systems for evoking strong antibody responses. Indeed, PPV-CPV and PPV-2CPV showed a high antigenicity and were efficiently recognized by anti-CPV antibodies in ELISA (Fig. 4), Western blot (Fig. 3) and immunomicroscopy (Fig. 5) analysis, emphasizing the utility of the PPV vector for diagnosis purposes. On the other hand, both chimeric viruses were immunogenic and able to induce high levels of antibodies in both mice and rabbits, but there seems not to be a substantial difference in the quality of immune response induced by PPV-CPV and PPV-2CPV. The elicited antibodies were able to neutralize CPV, although the neutralization titers were not very high. It is foreseen that further improvements of the system (modifications in the virus constructions and in the inoculation manner) may facilitate its use as a source of alternative vaccines.

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