Use of modified plum pox virus coat protein genes developed to limit heteroencapsidation-associated risks in transgenic plants

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Aphid transmission of a non-aphid-transmissible strain of zucchini yellow mosaic virus (ZYMV-NAT) occurs in transgenic plants expressing the plum pox potyvirus (PPV) coat protein (CP) gene. Heteroencapsidation has been shown to be responsible for this modification in the epidemiological characteristics of the infecting virus. In order to prevent this biological risk, several modified PPV CP constructs were produced that were designed to interfere with heteroencapsidation itself or to block aphid transmission of heteroencapsidated virions. These constructs were first expressed in Escherichia coli in order to check for the accumulation of pseudo-particles by electron microscopy. Virus-like particles (VLPs) were found with the full-length CP and with a PPV CP lacking the DAG amino acid triplet involved in aphid transmission. However, no VLPs were observed with CP lacking R220, Q221 or D264, amino acids known to be essential for the assembly of other potyvirus CPs. Transgenic Nicotiana benthamiana lines expressing the different PPV CP constructs were infected with ZYMV-NAT. Aphid transmission assays performed with these plants demonstrated that the strategies developed here provide an effective means of minimizing the biological risks associated with heteroencapsidation.

Introduction

The coat protein (CP) gene was the first transgene successfully used to confer virus resistance to transgenic plants (Powell et al., 1986). Since then, numerous examples of genetically engineered, CP-mediated plant virus resistance (CPMR) have been reported in various systems (herbaceous and woody plants), and against at least 13 different groups of virus (Beachy et al., 1990; Wilson, 1993; Grumet, 1994). The ‘quasi-universal’ efficiency of CPMR and initial reports describing some interesting cases of broad spectrum resistance (Stark & Beachy, 1989; Nejidat & Beachy, 1990) have made this strategy very attractive for the creation of virus-resistant crops. In 1995, the first transgenic squash resistant to zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus II was approved for commercialization in the USA.

Although tremendous progress has been made during the last 5 years in understanding the mechanisms involved in the resistance of transgenic plants expressing viral CP genes (for recent reviews see Lomonossoff, 1995; Baulcombe, 1996), only a few studies have been concerned with the assessment and control of biological risks which may be associated with such plants. The large-scale use of these plants could theoretically lead to new ecological problems (de Zoeten, 1991; Tepfer, 1993). In particular, the presence of transgene products (RNA or protein) in the same cell as an infecting homologous or heterologous virus could modify the epidemiological characteristics of the virus. Three phenomena could be involved in this modification: recombination, synergism and heteroencapsidation (Robinson, 1996).

Heteroencapsidation has long been recognized in mixed infections involving different groups of aphid-borne viruses that are transmitted in a persistent, semi-persistent or non-persistent manner (Rochow, 1972; Murant et al., 1976; Simons, 1976). Exchange of capsid subunits between viruses of the
same group can be responsible for the modification of vector specificity (Hu et al., 1988). In another case, a non-aphid-transmitted (NAT) strain became aphid-transmissible through phenotypic mixing (Bourdin & Lecoq, 1991).

Heteroencapsidation has also been observed between the CP produced in transgenic plants and the CP of incoming homologous or heterologous viruses (Osbourne et al., 1989; Farinelli et al., 1992; Lecoq et al., 1993). In the latter report, a ZYMV-NAT strain was transmitted by aphids when encapsidated with a plum pox potyvirus (PPV) CP synthesized in transgenic Nicotiana benthamiana plants.

In view of these results, our goal is to produce transgenic plants which reduce the biological risks linked to hetero-encapsidation. Newly modified PPV CP constructs were designed according to two strategies. The first was to remove the nucleotides coding for the DAG amino acid triplet involved in potyvirus aphid transmission (Atreya et al., 1990, 1991; Blanc et al., 1997). The new CP construct was designated ΔDAGCP (Jacquet et al., 1998). The second approach was to alter the CP transgene to obtain an encoded CP which would fail to assemble, thereby preventing possible hetero-encapsidation in transgenic plants. A bacterial expression system was first used to check for the ability of the mutant CP to form virus-like particles (VLPs). The potential biosafety afforded by the use of modified PPV CP genes was then assessed in transgenic plants.

Methods

- Modifications of the full-length PPV CP (FLCP) gene. The recombinant pBluescript plasmid pTP3 contained the FLCP cDNA sequence (Ravelonandro et al., 1992). Deletion of nucleotides coding for the DAG amino acid triplet was achieved by PCR. Production of pΔDAGCP has been described previously (Jacquet et al., 1998). Two other plasmids, in which the nucleotides encoding R\(^{219}\), Q\(^{221}\) or D\(^{241}\) were removed, were produced by site-directed mutagenesis as described by Kunkel et al. (1987). The mutagenic oligomers 5′ GCC AGA TAT GCC (**) TTT TAC GAA ATG ACT TCA ACG 3′ and 5′ GCC AAA CCC ACT TTT (**) ATT ATG GCA CGT TTC AGC GAC G 3′ were used as primers to yield the pΔCP and pΔQCP recombinant plasmids, respectively (asterisks in parentheses indicate the position of deleted triplets).

- Cloning and expression of the different CP genes in E. coli. A high-level expression system (Qiagen) was used to express the different CP constructs in E. coli strain SG13009 (pREP4). BamHI and BgIII restriction sites were inserted by PCR to flank each construct. Amplified fragments were then digested by these two enzymes and cloned in BamHI–BglII-digested pQE30 plasmid. The FLCP and modified PPV CP genes were thus unidirectionally cloned in the same frame as the ATG start codon of the vector. Moreover, a 6-His tag linked to the N-terminal part of each encoded protein can be used for protein purification. For each construct, three clones, verified by restriction mapping and sequencing, were tested by rapid screening of CP expression in small-scale (0.5 ml) cultures as described by the supplier. Three hours after induction with IPTG, cells were harvested by centrifugation and total soluble proteins were analysed on a 12% polyacrylamide gel (Laemmli, 1970). The highest expressing clone was selected for each construct.

- Extraction of different induced proteins under native conditions. Following the manufacturer’s standard protein extraction protocol, most of the induced proteins were insoluble. A novel protocol was thus developed to increase the yield of non-denatured recombinant proteins. Important changes that improved the yield were the use of very low concentrations of IPTG for the induction and addition of 0.2% Sarkosyl in the lysis buffer (Jagadish et al., 1991; McNally et al., 1991). These two factors allowed us to make about 95% of the induced CPs soluble without apparently affecting their ability to polymerize into VLPs. In the modified protocol, 100 ml culture was centrifuged (5000 g, 15 min) 4 h after induction with 0.1 mM (final concentration) IPTG. The pellet was washed twice in 10 ml cold STE buffer (20% sucrose, 100 mM Tris pH 8, 10 mM EDTA) and resuspended in 5 ml of the same buffer containing 1 mg/ml lysozyme. The cells were then incubated for 20 min at 4°C and centrifuged again (5000 g, 10 min). The spheroplasts were suspended in 3 ml lysis buffer (100 mM Tris pH 8, 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.2% Sarkosyl), gently shaken for 5 min and sonicated (three 10 s bursts in ice). RNase and DNase were added (1 µg/ml) for 20 min at room temperature before centrifugation (15 min, 11,000 g). The supernatant was collected and centrifuged again, the pellet was discarded and the supernatant was concentrated in a Speed-Vac until the volume reached about 300 µl. Aliquots from these samples were used for electron microscopy analysis.

- Immunosorbent electron microscopy. The procedure described by Bourdin & Lecoq (1991) was adapted for extracts from E. coli which were deposited on grids previously coated with a polyclonal PPV antiserum at a dilution of 1:3000. Immunosorbent electron microscopy studies on ZYMV-NAT-infected transgenic or non-transformed plants were conducted according to Lecoq et al. (1993).

- Cloning in a binary vector and plant transformation. The insertion of FLCP and ΔDAGCP genes between a promoter sequence and the NOS terminator has been described previously (Ravelonandro et al., 1992; Jacquet et al., 1998). The other modified PPV CP gene (3ACP) contained deletions of the nucleotides encoding the DAG triplet, R\(^{219}\), Q\(^{221}\) and D\(^{241}\). It was obtained by digesting the 5′ part of the ARQCP gene with BamHI and EcoRI and the 3′ part of the ΔDAGCP gene with EcoRI and Asp718. The fragments were then cloned into pBluescript, previously digested with BamHI and Asp718. The 3ACP gene was then inserted between the 35S cauliflower mosaic virus promoter and the NOS terminator. This cassette was in turn excised and cloned between the 35S cauliflower mosaic virus promoter and the NOS terminator. This cassette was then inserted in a binary plasmid pBi5ACP (Ravelonandro et al., 1992). The resulting plasmid is designated pBi5ACP. Primary transformants were produced by leaf disk transformation with Agrobacterium tumefaciens strain LBA4404 (Horsch et al., 1985). A schematic representation of the different constructs is presented in Fig. 1.

- Virus inoculation and detection. ZYMV-NAT does not readily move systemically in N. benthamiana (Gal-On et al., 1992). In order to increase the number of systemically infected plants, a first inoculation was performed on non-transformed N. benthamiana plants with sap extracts from infected ZYMV-NAT zucchinis. The presence of the virus in newly formed leaves was verified 15 days post-inoculation (p.i.) by double-antibody sandwich (DAS)–ELISA. Systemic leaves from ELISA-positive plants were collected and ground in a citrate buffer (1:3; w/v), and this extract was then used as an inoculum on 4-week-old transgenic or non-transgenic N. benthamiana. The leaves found to be ZYMV-NAT positive by ELISA, at 15 days p.i., were used for ZYMV-NAT aphid-transmission.

- Protein analysis. Accumulation of FL or modified PPV CP was evaluated by Western blot assay. Pellets from induced or non-induced SG13009 cells were suspended in lysis buffer (Laemmlli, 1970). Young
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N. benthamiana leaves were ground in the buffer described by Berger et al. (1989) at a dilution of 1:4 (w/v). Supernatant (10–30 μl) was electrophoresed on a 12% polyacrylamide gel (Laemmli, 1970) and blotted onto a nitrocellulose membrane. Polyclonal antibodies (1:1000) raised against PYYV CP were used to probe the different extracts.

**Aphid transmission experiments.** ZYMV-NAT-infected leaves of transgenic N. benthamiana from the lines BPC (Ravelonandro et al., 1992), 35A (Jacquet et al., 1998) or 3A, or from non-transformed plants, were used as source for aphid transmission. Non-viruliferous *Myzus persicae* were allowed an inoculation access period of 10 h on test plants before being transferred to test plants (15 or 50 aphids per plant). For each source leaf (two per plant), 20 test plants (V*°*rantais melons or Diamant zucchinis, depending on the experiment) were used. Aphids were allowed an inoculation access period of 10 h on test plants before being killed by an insecticide.

**Results**

**Effect of CP structure on formation of VLPs**

Bacteria were used to express FL and altered forms of PPV CP constructs and to study the effects of different amino acid deletions on the assembly of the CP into VLPs. The previously described FLCP and ADAGCP genes (Ravelonandro et al., 1992; Jacquet et al., 1998) and two newly modified PPV CP constructs (ARQCP and ΔDCP) were introduced into the expression plasmid pQE30 (Fig. 1). For ARQCP and ΔDCP nucleotides encoding the charged amino acids R18, Q21, and D68, located in the core of the PPV CP and shown to be involved in VLP formation of another potyvirus CP (Jagadish et al., 1991, 1993), were deleted.

 Cultures of *E. coli* transformed with pQFL, pQADAG, pQRQ or pQAD were induced with IPTG, and proteins were extracted and analysed by PAGE followed by Western blot. Fig. 2 shows the patterns of the different clones transformed with these plasmids. No extra band appeared in the pattern of induced SG13009 cells, which did not contain a pQE plasmid (lane 3). The PPV antiserum recognized bands of the expected size (36 kDa; Ravelonandro et al., 1992) for all four induced proteins (FLCP, ADAGCP, ARQCP and ΔDCP) (Fig. 2b, lanes 5, 7, 9 and 11).

**Extraction of the induced proteins and electron microscopy observations**

In order to observe assembly of the FL and of the modified PPV CP into VLPs, the proteins were extracted under non-denaturing conditions as described in the Methods; soluble protein extracts were concentrated and deposited on grids previously coated with a PPV antiserum. For each clone expressing the FLCP, ADAGCP, ARQCP and ΔDCP constructs,
10–20 grids were observed. Typical images are shown in Fig. 3. Flexuous PPV-like particles, 1–14 per grid square (Fig. 3a), were observed in the extracts prepared from FLCP and ΔDAGCP. These VLPs were highly heterogeneous in length (Fig. 3b) and were fully decorated by antiserum raised against PPV CP (Fig. 3d, e). VLPs were never observed on the 20 grids prepared with soluble protein extracts from induced cells containing the ΔRQCP and ΔDCP constructs.

Selection of T2 lines to perform risk assessment

A schematic representation of the different PPV CP constructs used to transform *N. benthamiana* is shown in Fig. 1. The production of pBIPCP and pB135ADAGCP, containing constructs encoding the FLCP and ΔDAGCP, respectively, has been described previously (Ravelonandro *et al.*, 1992; Jacquet *et al.*, 1998). Another modified PPV CP construct with three
This plasmid was designated pBI3. T2 lines homozygously transformed with the FLCP, DAS–ELISA. Surprisingly, the presence of the virus correlated with its resistance tests (Ravelonandro et al., 1993) and were used by Lecoq et al. (1998), (1993), except that a higher number of aphids (15 or 50 instead of 10) was transferred onto each test plant (see Methods). Once the presence of the ZYMV-NAT was confirmed, aphids were allowed to acquire the virus from these plants, before being transferred onto the cucurbit test plants for the inoculation access period. Nine independent experiments were first performed, using 15 aphids on each cucurbit test plant. Table 1 shows that ZYMV-NAT was transmitted only from BPC plants, with a rate of transmission of 2%, regardless of the test plant used (melon or zucchini), except for the third experiment in which no transmission at all occurred. On the other hand, virus was never detected in more than 400 test plants following the transfer of the aphids from infected transgenic plants harbouring the modified ΔDAGCP and 3ΔCP. In another experiment (data not shown), a higher number of aphids (50) was used per test plant in order to increase the transmission potential. Again, ZYMV-NAT was transmitted only from BPC plants, with a calculated transmission rate of 3.3% (3/90 test plants infected). No transmission occurred from 35Δ- or 3Δ-infected plants (0/75 test plants infected in each case).

The 19 test plants in which ZYMV-NAT was transmitted by aphids were used individually in secondary aphid transmission experiments to 10 zucchini plants with 10 M. persicae per plant. No transmission occurred, confirming that these plants were infected by ZYMV-NAT, whereas such an experiment conducted with an aphid-transmissible ZYMV isolate led to 100% transmission (Lecoq et al., 1991).

## Discussion

During the last decade, the CPMR strategy has been extensively studied and successfully used to produce virus-resistant transgenic plants (for reviews see Beachy et al., 1990;
Fig. 5. Phenotypic mixing observed in BPC (a) and in 35Δ (b) plants infected by ZYMV-NAT. Extracts from leaves used in the ZYMV-NAT aphid transmission assays were deposited on grids coated with a mixture of ZYMV and PPV antisera and were decorated with a PPV antiserum. Phenotypic mixing was observed in infected plants expressing the FLCP (a) and the ΔDAGCP (b). Bars, 200 nm.

Fig. 6. Lack of heteroencapsidated ZYMV-NAT particles in 3Δ plants. Each grid was coated with a mixture of ZYMV and PPV antisera. (a) No virus particles were decorated with PPV antiserum; (b) all particles were totally decorated with a ZYMV antiserum. Bars, 200 nm.
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Table 1. Number of infected cucurbit test plants after aphid transmission of ZYMV-NAT from transgenic or non-transformed plants

<table>
<thead>
<tr>
<th>Source plant</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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</tr>
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<td>BPC</td>
<td>2/90</td>
<td>2/100</td>
<td>0/80</td>
<td>2/99</td>
<td>2/96</td>
<td>2/80</td>
<td>1/80</td>
<td>3/100</td>
<td>2/70</td>
<td>16/795</td>
</tr>
<tr>
<td>35А</td>
<td>0/40</td>
<td>0/100</td>
<td>0/80</td>
<td>0/80</td>
<td>0/79</td>
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<td>0/70</td>
<td>0/10</td>
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<td>0/80</td>
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<td>0/80</td>
<td>0/79</td>
<td>0/68</td>
<td>0/406</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/10</td>
<td>0/170</td>
<td></td>
</tr>
</tbody>
</table>

BPC, 35А and 3А are transgenic plants expressing FLCP, ΔDAGCP or Δ3CP constructs, respectively. NT, Non-transformed plants. The presence of ZYMV-NAT in the source plants was tested by ELISA or Western blotting before each aphid transmission. Fifteen aphids were transferred onto each test plant. Zucchinis and melons were used as test plants in experiments 1–5 and 6–9, respectively.

Grumet, 1994; Baulcombe, 1996). However, the use of such a transgene could lead to potential modification of an incoming virus which could then acquire new biological characteristics. The existence of such a risk has been demonstrated by Lecoq et al. (1993): a ZYMV-NAT isolate could be aphid-transmitted through heteroencapsidation with an engineered PPV CP synthesized in transformed plants.

In this work, strategies have been developed and evaluated which could be used to avoid the risks associated with heteroencapsidation, while still preserving the initial interest of the CP transgene, e.g. production of pathogen-derived resistance. To prevent undesired effects, nucleotides coding for amino acids involved in aphid transmission (the DAG triplet) or thought to be essential for the assembly of the PPV CP (R<sup>220</sup>, Q<sup>221</sup> and Δ<sup>264</sup>) were deleted in these constructs. A prokaryotic system was first used to study the impact of the introduced deletions on the HP structure. Once produced in E. coli, the modified CPs were extracted under native conditions. VLPs, recognized by a PPV antiserum, were found in bacteria expressing the FLCP and ΔDAGCP genes. These observations are in agreement with the model proposed by Shukla et al. (1989) for the structure of the potyvirus CP, in that deletion of the N-terminal DAG triplet located on the surface of the particle did not affect the assembly of the CP subunits. However, VLPs were not observed in extracts from bacteria producing CPs with deletions of R<sup>220</sup>, Q<sup>221</sup> or Δ<sup>264</sup>, indicating that the presence of these amino acids is essential for assembly of the PPV CP. These results are consistent with the observations of Jagadish et al. (1991, 1993), who used a similar system to test the effects of deletions or substitutions of equivalent amino acids in the Johnsongrass mosaic virus CP. Dolja et al. (1994) obtained similar results when using tobacco etch potyvirus infectious clones with amino acid substitutions of these charged amino acids. Results concerning the assembly of different CPs in E. coli are corroborated by in planta results, obtained by searching for the presence of phenotypic mixing in transgenic plants synthesizing the various CPs after infection with ZYMV-NAT. Thus, the FLCP and ΔDAGCP, which were able to assemble in bacteria, could also partially or totally heteroencapsidate the ZYMV-NAT RNA. Such a phenomenon was never observed with Δ3CP, which failed to give VLPs in bacteria.

Western blot analysis following infection of transgenic BPC and 35А plants indicated that the presence of ZYMV-NAT was generally correlated with a visible increase in the accumulation of FLCP and ΔDAGCP. A similar increase in accumulation of a transgenic potyvirus CP following potyvirus infection has already been reported in transgenic potatoes by Farinelli et al. (1992), who suggested that the effect could result from a better stabilization of the protein, linked to its incorporation into particles of the infecting potato virus Y strain N. Such an explanation could also apply to our observations in BPC and 35А plants, given that no increase of the 3АCP level was observed in transgenic plants following ZYMV-NAT infection (not shown). The detection of larger amounts of engineered CP in a transgenic plant in the presence of a heterologous potyvirus could thus represent additional evidence for the occurrence of phenotypic mixing.

This report confirms the results of Lecoq et al. (1993) showing that the PPV FLCP can complement the deficient ZYMV-NAT strain through heteroencapsidation. However, despite analysis of more than 400 test plants on which aphids were transferred from infected 35А or 3А plants, no ZYMV-NAT transmission was detected. These results thus show that the deletions introduced in the modified PPV CPs have allowed heteroencapsidation-associated risks to be controlled.

Although a large number of heteroencapsidated ZYMV-NAT particles were observed in the 35А plants, no aphid transmission occurred. This result confirms the recent demonstration that the DAG triplet, located in the amino part of the CP, is directly involved in potyvirus aphid transmission through interactions with the helper component (Blanc et al.,...
1997). A second DAG triplet is present in the PPV CP, as in some other potyviruses. As no complementation occurred with 35A plants, this second DAG is apparently not functional, probably due to its unexposed location in the CP. Newly modified PPV CP constructs have been produced to make the second DAG accessible. Transgenic plants expressing such constructs will be tested for aphid transmission of ZYMV-NAT to determine whether the second DAG can be rendered active.

Although recombination has been shown to occur between potyviruses (Revers et al., 1996) or between a transcript and a homologous incoming virus (Greene & Allison, 1994), the negative results obtained during secondary aphid transmission experiments suggest that no recombination has occurred between ZYMV-NAT RNA and PPV CP transcripts. Unlike other reports, in which recombination was described between a transgene-derived transcript and a viral RNA (Lommel & Xiong, 1991; Greene & Allison, 1994; Falk & Bruening, 1994), only a weak selection pressure was applied to ZYMV-NAT. Indeed, neither virus replication nor movement was blocked in the transgenic plants expressing PPV CP genes. These conditions could thus explain why no recombination was detected between the ZYMV-NAT RNA and the sequence encoding the DAG triplet from the FLCP transcripts.

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References


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