Peptide display on Potato virus X: molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles

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The potexvirus Potato virus X (PVX) can be modified genetically to generate chimeric virus particles (CVPs) carrying heterologous peptides fused to coat protein (CP) subunits. A spontaneous PVX mutant expressing a truncated, but functional, form of the CP has been isolated. With the aim of exploiting this virus to display peptides useful for vaccine formulations, two novel viral expression vectors based on pPVX201 (bearing the wild-type PVX genome) were constructed encoding the truncated CP. Both vectors were able to produce infectious virus particles in planta and were used to insert a panel of sequences encoding peptides of biopharmaceutical interest as N-terminal fusions to the truncated cp gene. The analysis of infection progression induced by the different constructs enabled identification of two important structural features of the fused peptide, namely tryptophan content and isoelectric point, critically affecting the formation of PVX CVPs and virus movement through the plant. These results are discussed in view of the rising interest in engineered plant viruses for development of peptide-based epitope vaccines.

INTRODUCTION

Genetically modified plant viruses can be used to transiently express heterologous sequences in susceptible plant hosts (Pogue et al., 2002). A promising application of engineered plant viruses is the development of epitope vaccines by the generation of chimeric virus particles (CVPs) that display peptides of interest on their surface as fusions to the coat protein (CP) (Scholthof et al., 1996). The potexvirus Potato virus X (PVX) has been shown to be an ideal, highly ordered, multivalent scaffold to be used for this purpose (Brennan et al., 1999; Marusic et al., 2001). PVX could also be used as a carrier of whole proteins, although this approach presents some disadvantages in vaccine applications due to the unpredictability of the number of chimeric CP units per virion, precluding an exact determination of the vaccine dose (Santa Cruz et al., 1996).

PVX filamentous particles consist of a single-stranded, positive-sense RNA molecule embedded in a capsid made of approximately 1300 units of the same protein. Although no atomic-resolution data are available defining the organization of the virus particle and CP folding, it is assumed that PVX has a helical structure, with the CP N terminus exposed on the viral surface (Baratova et al., 1992a, b; Parker et al., 2002). For this reason, the construction of PVX CVPs requires the fusion of exogenous sequences at the 5′ terminus of the cp gene. The genetic manipulation of PVX has been greatly improved by the development of expression vectors, such as pPVX201, that contain the cDNA encoding the complete viral genome (Baulcombe et al., 1995; Chapman et al., 1992a).

In this work, we have isolated a spontaneous PVX mutant characterized by a deletion at the 5′ terminus of the cp gene. Two pPVX201-derived vectors encoding the cp mutant gene have been constructed and were used to fuse, to the 5′ end of the cp gene, sequences encoding a panel of peptides varying in both length and amino acid composition. From the observation of the infectious phenotypes induced in planta by the different constructs, we concluded that cell-to-cell and phloem movement of PVX CVPs is critically affected by the occurrence of tryptophan (Trp), as well as the isoelectric point (pI) of the peptide.
METHODS

DNA constructs. Construction of the pPVX201 viral expression vector (kindly provided by D. Baulcombe, The Sainsbury Laboratory, Norwich, UK) (Baulcombe et al., 1995) encoding the peptide KSS (amino acid sequence CKSGKLISSL) (Scala et al., 1999) as an N-terminal fusion with the CP was performed as described previously (Marusic et al., 2001), adopting a strategy that removes the duplicated cp subgenomic promoter.

To construct the viral expression vector pPVXCC, the 5’-deleted portion of the mutant cp gene was substituted to the wild-type cp in pPVX201. Briefly, an RT-PCR fragment was obtained by using total RNA extracted from the systemically infected leaves of pPVX201-KSS-inoculated plants as a template and primers PVXBack (5’-CTGGGGAAATCAATCACTGTTG-3’) and PVXxho (5’-GACTGGATTATGGTGGTGTAG-3’). The PCR product was digested with the restriction enzymes NheI and XhoI and ligated into similarly digested pPVX201. To construct the viral expression vector pPVXSm, in which sequences recognized by the restriction enzyme Smal were inserted immediately downstream of the start codon of the mutant cp gene, sense (5’-TGCACTACGGGCGGACTCTGGCCACAGCTCA-GG-3’) and antisense (5’-CTGAAGCTGGATCCAGGTTGCG-GGATGTAG-3’) primers were designed and annealed in vitro to generate a fragment with Nhel-compatible 5’ and StuI-compatible 3’ ends. This fragment was ligated into Nhel-StuI-digested pPVXCC.

To insert sequences encoding the heterologous peptides as 5’-end fusions with the cp gene in pPVXCC, primer pairs were designed (following the PVX cp codon usage) and annealed in vitro to obtain fragments with Nhel-compatible 5’ and StuI-compatible 3’ ends to be ligated into the Nhel-StuI-digested vector. All of the sequences encoding the peptides were preceded by an ATG codon and followed by the cp gene portion that is lost by StuI digestion.

To insert the sequences encoding the heterologous peptides as fusions with the cp gene into pPVXSm, oligonucleotide pairs were designed to obtain Nhel-compatible 5’- and Smal-compatible 3’-end DNA fragments that were ligated into the Nhel-Smal-digested vector. As reported previously, sequences encoding the peptides were preceded by an ATG codon.

The biochemical features of the N-terminal CP peptides encoded by pPVXCC- and pPVXSm-derived constructs were calculated by using the ProtParam tool (http://www.expasy.org/tools/protparam.html).

Plant infection. Nicotiana benthamiana plants were inoculated with the different constructs or with pPVX201 (as control) as described previously (Marusic et al., 2001). To verify the genomic stability and infectivity of virus particles that cause systemic infection, repeated cycles of reinfection were performed using leaf sap, prepared by using an Ultracut-E ultramicrotome (Reichert–Jung) were collected on copper grids and stained with 1 % (w/v) uranyl acetate solution. Sections were then analysed by using a transmission electron microscope EM208 (Philips).

RESULTS

Isolation of a PVX CP mutant

The PVX viral expression vector pPVX201 (strain X3) (Fig. 1a, b) was modified by inserting at the 5’ end of the cp gene the sequence encoding a peptide of interest (KSS) for vaccine formulations. The resulting construct, pPVX201-KSS, was used to inoculate N. benthamiana plants with the aim of obtaining CVPs displaying the KSS peptide on their surface. Ten days p.i., the systemic leaves showing vein chlorosis (a typical symptom of PVX infection) were sampled. To verify the presence of the expected modified viral genome within the leaves, total RNA was extracted, reverse-transcribed and amplified in the region including the 5’ end of the cp gene. Surprisingly, we found a rearrangement of the cp gene, causing the loss of nt 4–67 from the 5’ end. Analysis of the deduced amino acid sequence revealed that residues 2–22 at the N terminus were lost and substituted by a cysteine (Cys) residue in the correct reading frame.

Construction of pPVXCC and pPVXSmA viral expression vectors

The rearranged cp gene was substituted to the wild-type cp in pPVX201, generating the pPVXCC viral expression vector (Fig. 1d). To verify the ability of pPVXCC to produce infectious virus particles, the vector was used to inoculate N. benthamiana plants. Ten days p.i., systemic leaves of the plants displayed the same symptoms induced by pPVX201 with PVXBack and PVXNew (5’-CAGTCTAGCTGCTGATGC- GTTG-3’) primers. PCR fragments were purified and verified by sequencing.

Western blot analysis and ELISA. Protein extracts were obtained from inoculated and systemic leaves as described previously (Marusic et al., 2001). Aliquots (12.5 µg for extracts of symptomatic leaves or 20 µg for extracts of asymptomatic leaves) were separated on a 12.5 % (w/v) SDS-PAGE gel before transferring onto a PVDF membrane (Millipore). Immunodetection was performed as described previously (Donini et al., 2005).
adding at the 5’ end of the deleted cp gene a unique restriction site (SmaI) (Fig. 1f). This modification produced the substitution of Cys (CP position 2) and alanine (Ala) (CP position 3) with proline (Pro) and Gly, respectively (Table 1). The resulting pPVXSma vector induced symptoms on systemic leaves that were identical to those of the controls (Fig. 1g). The stability of the genome of these virus particles was verified as described previously (data not shown).

**Heterologous peptides**

To determine peptide traits influencing structural stability and infectivity of the chimeric viruses, peptides of different length and amino acid composition were chosen to be displayed as CP fusions (Table 1). The panel of peptides included epitopes selected from melanoma-associated (MART, MARTSh, NYESO, NYESOSh) (Kirkin et al., 1998) or human immunodeficiency virus type 1 (Z13, Z13Cys, 2F5L, 2F5LCys, FZ, FZSh, FZShCys, SL9, P18, NefB, NefC) (Hurwitz et al., 2005; Shiver & Emini, 2004) proteins. Other peptides, associated with coeliac disease (EB1, EB2, EB3) (Shan et al., 2002) or endowed with antimicrobial activity (PK, SK) (Donini et al., 2005; Polonelli et al., 2003), were also included. To elucidate a possible role of the Cys residue in position 2 of pPVXCC CP on viral stability, some of the peptide-encoding sequences were designed both with and without this amino acid in this position.

**Symptoms and molecular analysis of plants inoculated with the chimeric CP-encoding constructs**

Ten to twelve days p.i., systemic leaves were examined to evaluate the presence of virus symptoms. Subsequently, plant tissues were analysed for the presence of the expected viral RNAs and CPs by sequencing and Western blotting. The constructs were divided easily into two main groups: those able to induce systemic infection (pPVXSma-SL9, pPVXSma-EB1, pPVXSma-EB2, pPVXSma-EB3, pPVXSma-MARTSh, pPVXSma-KS, pPVXSma-SK, pPVXSma-NefB, pPVXSma-NefC) and those that were not. Viral RNA was present in systemic leaves of plants inoculated with constructs able to induce systemic infection (group I; Fig. 2a). Although major sequence rearrangements did not occur, occasional point mutations affected the heterologous nucleotide sequence selectively, resulting in amino acid substitutions in the displayed peptide. This phenomenon was mostly evident during reinfections, but in one case (pPVXSma-EB1), the substitution occurred during the infection cycle produced by plasmid inoculation (Table 2). In view of the possible application of the systemically moving and genetically stable CVPs to biopharmaceutical research, some of them were produced on a large scale in plants and, after purification, the nature of CP–peptide fusion proteins was ascertained by extensive mass mapping experiments (see supplementary material, available in JGV Online).
Table 1. List of the non-consensus N-terminal sequences of the unmodified or chimeric mutant CP putatively encoded by each construct.

The table is a list of the non-consensus N-terminal sequences of the unmodified or chimeric mutant CP putatively encoded by each construct and considered in the subsequent analysis. The Thr residue in parentheses is the first amino acid residue common to all of the encoded CPs, not considered as belonging to the N-terminal peptides. The amino acid sequences indicated in bold are those of the heterologous peptides, whilst amino acids indicated in plain text are non-consensus residues of the mutated or wild-type CP. The table includes the N-terminal portion of the CP encoded by pPVX201 (23 aa) and pPVX201-2F5 (27 aa) constructed in our laboratory (Marusic et al., 2001) that have both been considered in the statistical analysis.

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<th>Fusion construct name</th>
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<th>W (%)†</th>
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<td>+/+</td>
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*Isoelectric point.
†Percentage content of Trp.
‡Presence (+) or absence (−) of symptoms on inoculated and systemic leaves.

The initial subdivision of the constructs into two groups, based only on the analysis of the systemic leaves, was extended when inoculated leaves were also evaluated. Constructs unable to induce the systemic infection could be split into a group able to induce local lesions on inoculated leaves (i.e. necrotic ringspots) (pPVXSm-A, pPVXSm-MARTCys, pPVXSm-P18) (group II; Fig. 2b) and a group unable to induce symptoms (pPVXCC-Z13, pPVXCC-Z13Cys, pPVXCC-2F5L, pPVXCC-2F5Lcys, pPVXCC-FZ, pPVXCC-F2Sh, pPVXCC-F2ShCys, pPVXSm-NYESOSh, pPVXSm-NYESO) (group III; Fig. 2c). ELISA analysis of the leaves inoculated with constructs belonging to group III revealed that, although local spreading was not detectable, these constructs were able to direct the synthesis of CP (data not shown).

Effect of amino acid composition of the heterologous peptide on genetic stability and on viral cell-to-cell movement

With the goal of defining general rules for the rational design of chimeric CPs in PVX, attributes correlating with the
The in vivo performance of each group of chimeric CPs were evaluated. We considered as heterologous peptide every non-consensus N-terminal sequence extending over the threonine (Thr) residue in position 24 of the CP encoded by pPVX201 (Table 1). This allowed a comparative analysis of pPVXCC-, pPVXSma- and pPVX201-derived constructs. The first trait to be analysed was peptide length, as the capability of generating assembled virus particles able to infect plants systemically could have been related primarily to the extent of peptide steric hindrance. Peptides were thus ordered by length and considered in terms of the in vivo behaviour of the CP–peptide fusion-encoding construct. As shown in Fig. 3 (upper panel), this criterion did not consistently explain the overall performance of our constructs in planta, as group I, II and III constructs extended randomly over the different peptide lengths (10–24 aa).

The second trait to be analysed was the amino acid composition. In particular, we focused on the percentage content of each amino acid within the heterologous peptide. The presence/absence of the Cys residue at position 2 did not affect in planta performance. In addition, the analysis of group I-encoded peptides revealed that there was not a particular bias towards a specific composition compared with the other groups and that these constructs mainly accumulated point mutations in the heterologous peptide-encoding sequence when an N-terminal Pro residue and/or no or a very low serine (Ser)/Thr content was present (Table 2). Although definite amino acid residues have not been identified as strictly necessary for CVP systemic movement, we observed that Trp occurred only and always in peptides encoded by non-permissive group III constructs (Table 1). The only exception was the permissive construct pPVX201-2F5, which, however, showed a significantly low Trp content (3–7 %) when compared with the lowest value (6–2 %) of group III-encoded peptides. To demonstrate that Trp may hinder virus movement, we designed a Trp to Gly substitution in the peptides encoded by pPVXSma-NYESO (Trp content 6·2 %) and pPVXSma-NYESOSH (Trp content 10 %) group III constructs by site-directed mutagenesis. The new chimeric constructs, named pPVXSma-NYESOM and pPVXSma-NYESOSHM, were evaluated in their ability to induce local and/or systemic symptoms. By removing Trp, both mutated constructs were able to induce local and systemic lesions, acquiring the characteristics of group I constructs (Fig. 4).
**Effect of isoelectric point of the N-terminal peptide on viral systemic movement**

Further analysis was carried out on the correlation between viral infection and the pI value of the heterologous peptides. By ranking the peptides according to their pI value, we found that all of the constructs able to induce systemic infection (group I) encoded CP N-terminal peptides with a pI value within a defined range (5.24–9.18) (Fig. 3, lower panel). In contrast, peptides encoded by group II and group III constructs had a pI value outside this range. The only exceptions were the non-permissive group III constructs pPVX-Sma-NYESO and pPVX-Sma-NYESOSh, encoding peptides characterized by a high Trp content (Table 1).

To prove that PVX systemic movement (i.e. access to the phloem) is somehow affected by the pI of the displayed peptide, the pI values of the peptides encoded by two group II constructs (pPVX-Sma-p18 and pPVX-Sma-MART) and by one group III construct (pPVX-Sma-Z13) were modified. Modification to a permissive pI value was achieved by cloning, into pPVX-Sma, fragments designed to append one or two suitable amino acid residues to the ends of the original peptides, thus preserving their immunological properties. The sequence of the peptide P18 (pI 12.00) was modified by adding to each end an aspartic acid residue (Asp), generating the peptide P18DD (pI 5.96); the sequence of the peptide MART (pI 3.80) was modified by adding to each end a lysine residue (Lys), generating the peptide MARTKK (pI 6.14); finally, the sequence of the peptide Z13 (pI 3.80) was modified by adding to the C terminus a Lys residue, generating the peptide Z13K (pI 5.84) (Table 1). The inoculated and systemic leaves of the plants inoculated with the new constructs (pPVX-Sma-P18DD, pPVX-Sma-MARTKK and pPVX-Sma-Z13K) were examined carefully for the presence of symptoms and harvested for molecular analysis. Whilst no effect was observed for the construct originally belonging to group III, modification in group II constructs generated functional CVPs able to move systemically, despite the increase in peptide length (Figs 3 and 4).

**Electron microscopy analysis of the leaves inoculated with pPVX-Sma-p18 and pPVX-Sma-p18DD**

To understand how the pI of the peptides fused N-terminally to the CP could affect systemic spreading, leaves of the plants inoculated with the vector pPVX-Sma and with the constructs pPVX-Sma-P18 and pPVX-Sma-P18DD were analysed by electron microscopy. Analysis was focused on the lesions of inoculated leaves. The results revealed that pPVX-Sma-P18DD was able to induce not only the laminated inclusion components (LICs) (Shalla & Shepard, 1972) typical of PVX, but also large, cytoplasmic, fibrous aggregates of assembled virus particles (Lesemann, 1988) (Fig. 5b). Conversely, these virus-particle aggregates were not identified in the local lesions induced by pPVX-Sma-P18, which were characterized only by the presence of LICs in the cytoplasm of the infected cells (Fig. 5c, d).

**DISCUSSION**

In this work, we describe the display of peptides of biopharmaceutical interest on a stable PVX mutant containing a truncated CP. The mutant virus was chosen
mainly because it is hypothetically more suitable for the display of longer peptides than the wild type, but also as it is putatively less immunogenic, due to reduced CP dimensions (Koenig & Torrance, 1986). This could be an important feature when chimeric PVX is used as carrier of vaccine peptides, an application that requires repeated administration (Borisova et al., 1999; Chapman et al., 1992a, b) through both plasmodesmata and phloem (Fedorkin et al., 2001; Santa Cruz et al., 1998). By the visual inspection of inoculated plants, the chimeric constructs were classified into three main groups. In the case of groups I (symptoms on both inoculated and systemic leaves) and II (symptoms only on inoculated leaves), the infection phenotype matched perfectly with the molecular analysis performed. On the contrary, group III constructs, despite being unable to induce detectable symptoms, were able to direct the synthesis of the CP in the inoculated leaves, although at low levels. This result demonstrated that the performance of these constructs could not simply be ascribed to transcriptional problems, but rather to a functional failure of the chimeric CP that prevents the transport of the viral genome from initially infected cells to the neighbours.

Because group III peptides limited viral local movement, whereas group II peptides were able to hinder CP function selectively in phloem transport, our data indicate that the CP exerts distinct functions in cell-to-cell and systemic movement. To shed light on how peptide attributes affect each function selectively, we tried to identify common physicochemical parameters that characterized each group of peptides unambiguously. Ranking peptides according to their length resulted in a random distribution of the three groups, indicating that this parameter was unrelated to ability (group I) or not (group II and group III) to generate viral forms moving systemically. Peptide groupings were further analysed in terms of amino acid composition. This approach revealed that, although systemic movement was not related to a specific amino acid composition, the presence of Trp residues was peculiar to group III peptides. One exception to this rule was pPVX201-2F5 (group I), yet encoding the peptide with the lowest Trp content. Trp residues are often found associated with the end of hydrophobic regions in transmembrane proteins and are supposed to act as ‘floats’ at the membrane boundary, by fixing the protein to the lipid bilayer (Lee, 2003). On this basis, it could be hypothesized that the inability of group III constructs to promote cell-to-cell spreading might be ascribed to peptide-mediated membrane anchoring of chimeric CP, inhibiting cell-to-cell transfer. This hypothesis was supported by the fact that group III peptides were mainly putative transmembrane or membrane-proximal peptides (Chen et al., 1997; Salzwedel et al., 1999) and was

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**Fig. 4.** Effect of Trp→Gly substitution and of pH value modification on viral cell-to-cell and systemic movement, respectively. Left side, inoculated (I) and systemic (S) leaves of plants inoculated with the original constructs; right side, inoculated (I) and systemic (S) leaves of plants inoculated with the constructs encoding peptides bearing the Trp→Gly substitution or the pH modification.
finally confirmed by site-directed mutagenesis experiments showing that Trp to Gly substitution completely restored the ability of the CP to induce systemic infection.

Another correlation found to be highly significant was between the pI range of group I peptides and systemic movement. The robustness of this correlation was tested by modifying the pI value of peptides encoded by group II and group III constructs (i.e. MART, P18 and Z13). The results of these experiments showed that, whilst pI refinement of group II peptides resulted in the generation of viral forms able to move systemically (hence acting as group I peptides), pI adjustment of group III peptides had no effect. These data support the idea that the influence of pI on systemic infection may be hindered by a particularly high Trp content, interfering with earlier stages of viral movement. Thus, we can conclude that the effect of pI is selective on systemic movement, whilst Trp affects cell-to-cell movement independently of the pI value.

Despite the fact that all constructs belonging to group I conformed to the Trp and pI 'rules', some of them were subjected to point mutations in the sequences encoding the heterologous peptides. These mutations occurred mainly during reinfection cycles and were mainly biased towards the increase of Ser/Thr content, supporting findings that identify in these residues the phosphorylation sites involved in virus unpackaging (Baratova et al., 2004; Kozlovsky et al., 2003).

Electron microscopy studies, aimed to define differences between group II and pI-remodelled group I constructs, showed that whilst LICs were present in both samples, formation of virus-particle aggregates in infected cells was evident only after pI value adjustment. These results indicate that a non-permissive pI value (i.e. a charged N terminus) could interfere with the correct assembly of chimeric CP subunits to form complete virions. It is indeed a common belief that the introduction of repulsive charges in the N-terminal domain through phosphorylation is associated with PVX disassembly (Atabekov et al., 2001; Kozlovsky et al., 2003).

The major drawback to the production of CVPs harbouring peptides of interest in plants has been ascribed primarily to the steric interference of the foreign peptide with the correct assembly of virus particles (Scholthof et al., 1996), but peptide pI/charge has also been shown to affect viral fitness. In the case of rod-shaped Tobacco mosaic virus, this parameter affects cell-to-cell movement and this phenomenon has been ascribed to a lethal effect of the CP–peptide fusion on host cells (Bendahmane et al., 1999). Conversely, in both icosahedral Cowpea mosaic virus and filamentous Zucchini yellow mosaic virus, the effect is on systemic movement and is explained in terms of interference in the interaction between the CP and undefined host components (Kimalov et al., 2004; Porta et al., 2003). From these data, it is evident that common physicochemical features of a displayed peptide can influence the fitness of diverse viral
genera (i.e. Tobamovirus, Comovirus and Potyvirus), but that the effect is different depending on the different role played by virus particles and coat proteins in the virus life cycle. This is further confirmed by our work that has extended the knowledge base of the effects of peptide display to the filamentous potexvirus PVX. Our findings, by providing new insights into the prediction of peptide sequences compatible with the production of infectious CVPs for candidate vaccines, reinforce the notion that PVX moves from cell to cell and through the phloem in two structurally different forms (i.e. cell-to-cell movement as a ribonucleoprotein complex and systemic movement as assembled virus particles) (Lough et al., 1998, 2000).

From the current and prior studies, we can conclude that information derived from peptide-display technology applied to viruses of different phylogenetic origins has important implications both to produce candidate vaccines and to deepen knowledge of virus biology.

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