Antigen Delivery Systems III: Use of Recombinant Plant Viruses

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The most widely used approach to the expression of foreign proteins in plants is stable genetic transformation. This involves integration of heterologous genes into the chromosomes of a host plant and has been used successfully to express a number of immunologically active proteins (see Chapter 58). An alternative approach involves the use of plant virus–based vectors. One of the main attractions of this approach is that viral genomes multiply within infected cells, potentially leading to very high levels of protein expression. Additional advantages include the fact that viral genomes are very small and therefore relatively easy to manipulate and that the infection process is simpler than transformation/regeneration. There are, inevitably, disadvantages to the approach: the foreign gene is not heritable, there are limitations on the size and complexity of the sequences that can be expressed in a genetically stable manner, and there are concerns about the ability of modified viruses to spread in the environment. Nonetheless, the virus vector approach has been used to express a number of immunologically active proteins, and this article reviews the progress in the field and problems that remain to be solved.

TYPES OF VIRAL VECTOR

The first plant viruses to be investigated as potential gene vectors were those with DNA genomes. However, for a number of reasons, these proved difficult to develop into practical vectors for large-scale protein expression, and they have not been used for the production of immunologically active proteins. Details of the development and applications of DNA virus–based vectors are therefore outside the scope of the current chapter, and the reader is referred to Porta and Lomonossoff (1996, 2002) for a discussion of these systems.

Most plant viruses have genomes that consist of one or more strands of positive-sense RNA. These viruses can grow in a wide range of hosts, and some can reach extremely high titers. As with animal RNA viruses, they use a variety of strategies for gene expression, including the use of subgenomic promoters and polyprotein processing. The availability of infectious cDNA clones was prerequisite for the development of RNA virus–based vectors. Since their advent, members of several virus families have been developed as useful vectors **(Fig. 59.1)**. Initial attempts at vector construction were based on gene replacement strategies in which a sequence encoding a non-essential viral function was replaced by a gene of interest. Though several of these early constructs could replicate in isolated plant cells, they generally could not systemically infect whole plants. A more successful approach has been the development of vectors based on gene addition. In these a foreign sequence is added to the complement of viral genes, rather than substituting for one. For reviews of the development of RNA virus-based vectors the reader is referred to Scholtof *et al.*(1996), and Porta and Lomonossoff (1998, 2002).

Two basic types of systems have been developed for the production of immunogenic peptides and proteins in plants. The first type, often termed epitope presentation, involves inserting a sequence encoding an antigenic peptide into the viral coat protein gene in such a manner that the peptide is expressed on the surface of assembled virus particles. The modified virions are often referred to as chimeras or chimeric virus particles. Such particles are attractive as potential novel vaccines, since the presentation of multiple copies of an antigenic peptide on the surface of a macromolecular assembly can significantly increase its immunogenicity (Lomonossoff and Johnson, 1996). With these systems it is generally anticipated that the modified particles will be at least partially purified prior to administration to animals. The second type, often referred to as polypeptide expression, involves introducing a whole gene into the viral genome in such a manner that it is efficiently expressed in infected cells, usually as an unfused polypeptide. Though purification of the expressed protein may be necessary or desirable, this type of system could be suitable for the production of
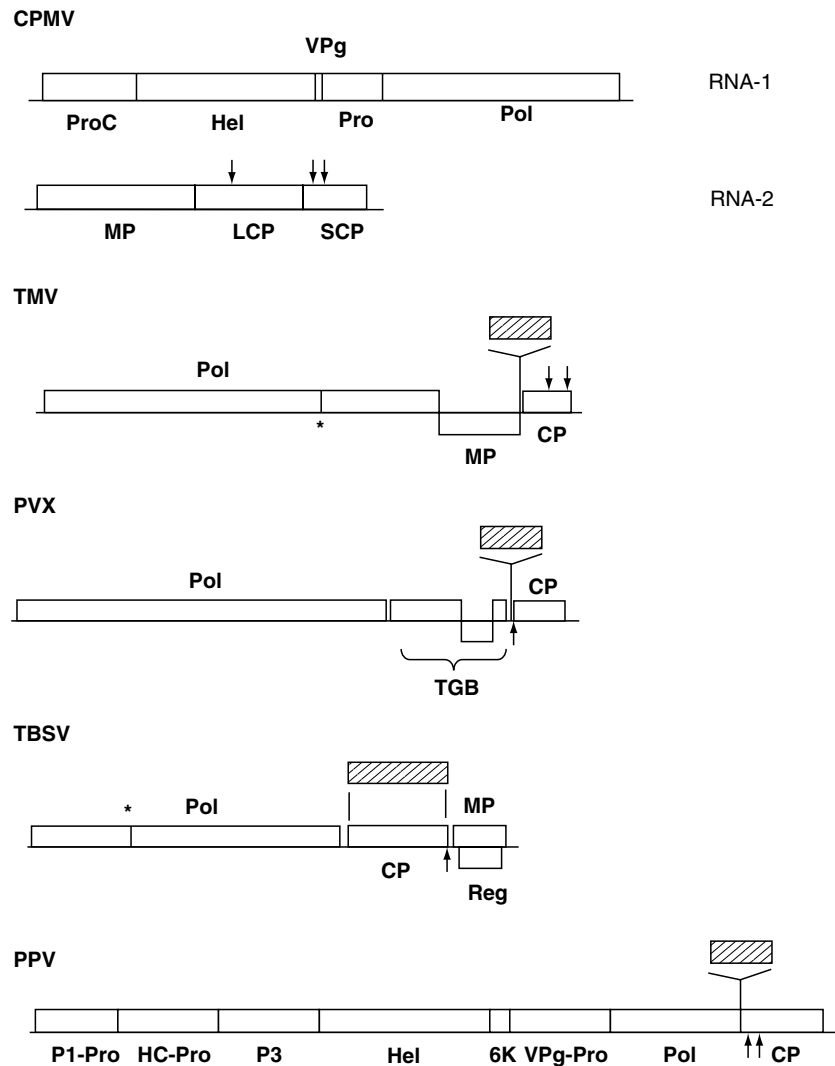
CPMV

Fig. 59.1. Genome organization of viruses used to express heterologous peptides and proteins in plants. Positions where epitopes have been inserted into the coat proteins of the various viruses are shown by black arrows. The positions where foreign proteins (shown hatched) have been inserted into the viral genomes are also indicated. The functions of various virus genes are shown as follows: CP, coat protein; HC-Pro, helper component proteinase; Hel, helicase; MP, movement protein; LCP, large coat protein; Pol, RNA-dependent RNA polymerase; Pro, proteinase; ProC, proteinase cofactor; Reg, regulatory protein; TGB, triple gene block; VPg, virus protein genome-linked; P1-Pro, P1-proteinase; P3, protein P3; 6K, 6-kDa protein; SCP, small coat protein; and VPg-Pro, VPg-proteinase. The *asterisk* represents a leaky termination codon.

immunogens that can be supplied orally by direct feeding of plant material to animals.

EPITOPE PRESENTATION SYSTEMS

A number of viruses of various morphologies have been adapted for use as epitope presentation systems. The main prerequisites for such a use are that the presence of the foreign sequence does not interfere with ability of the modified coat protein to assemble into virions and that the peptide is displayed on the surface of assembled particles. Thus, attention to date has focused on those viruses for which there is at

least some information available about the topology of the coat protein in the assembled virions. As more structural information accumulates, it is likely that additional plant viruses will be developed into epitope presentation systems.

Cowpea mosaic virus (CPMV)
Construction of CPMV chimeras

Cowpea mosaic virus (CPMV) was the first plant virus to be developed as an epitope presentation system (Usha *et al.*, 1993; Porta *et al.*, 1994, 1996). CPMV is a bipartite RNA virus (Fig. 59.1), with particles containing 60 copies each of a large (L; 37 kDa) and a small (S; 23 kDa) CP arranged with icosahedral symmetry **(Fig. 59.2)**. The virus
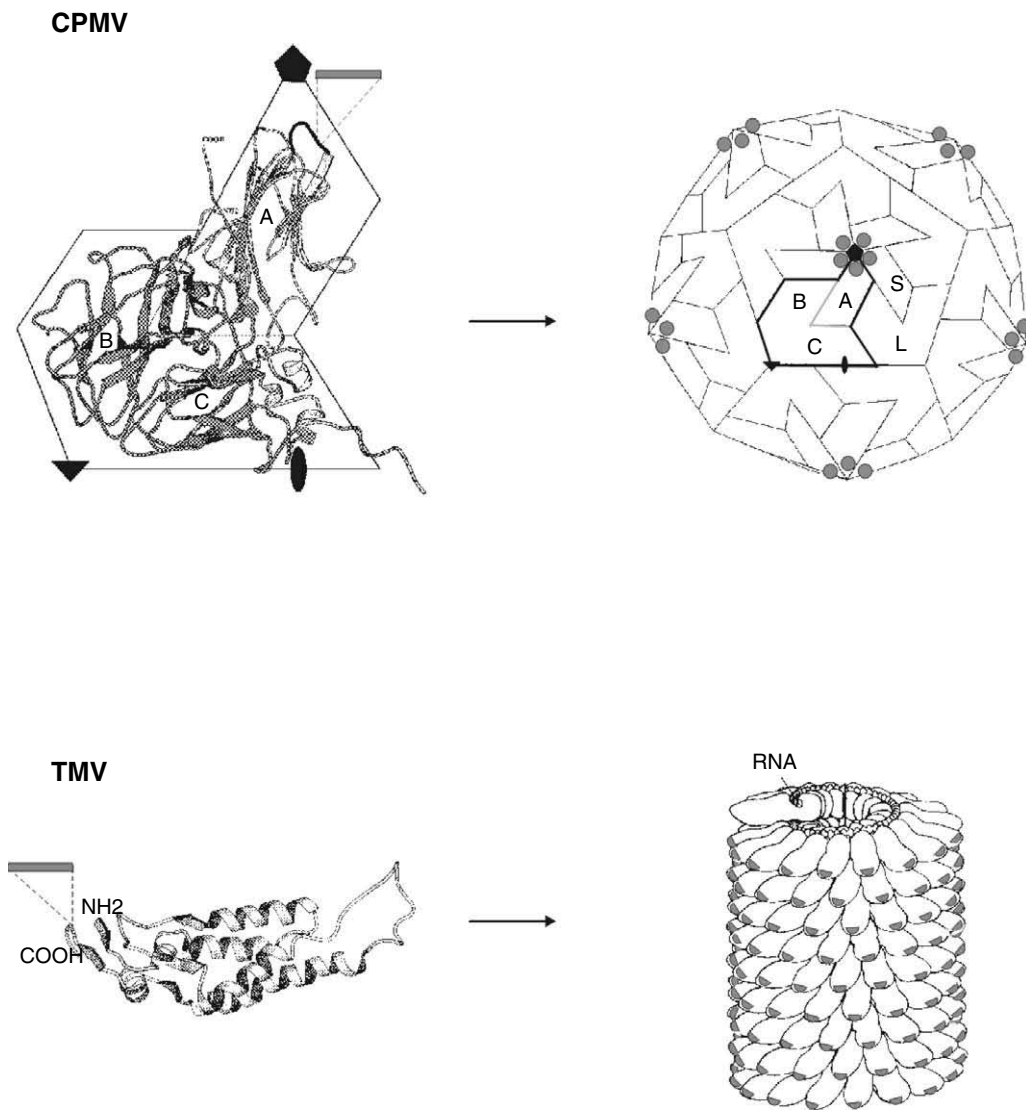
CPMV

TMV

Fig. 59.2. Epitope presentation using CPMV (*top*) and TMV (*bottom*). CPMV: *left,* the assymetric unit of the virus. This consists of one copy each of the large (L; C and B domains) and small (S; A domain) coat proteins. The site most commonly used for the insertion of foreign peptides, the βB-βC loop of the S coat protein, is indicated. The icosahedral particles *(right)* contain 60 copies each of the S and L coat proteins, and the positions on assembled virus particles of an epitope inserted in the βB-βC loop of the S coat protein are shown as *grey dots*. TMV: *left,* the position on the coat protein subunit near the C-terminus where insertions have been made in most chimeras. The approximate positions of inserted epitopes are shown as *shaded dots* on a segment of an assembled virus particle (*right*).

was an attractive candidate for development as an epitope presentation system because it grows to high titers and the detailed three-dimensional structure of the coat protein was known. This enabled a rational choice to be made regarding potential insertion sites (Lomonossoff and Johnson, 1995). Since the original reports describing the construction of chimeric virus particles, a large variety of epitopes have been expressed on the surface of CPMV particles (for examples, see Lomonossoff and Hamilton, 1999, and Porta *et al.*, 2003). In most cases, the foreign sequence has been inserted into the most exposed loop of the virus surface, the βB-βC loop of the S protein (Fig. 59.2). However, other sites, such as the βE-αB loop of the L pro-

tein and the βC′-βC″ loop of the S protein, have also been used successfully (Brennan *et al.*, 1999a; Taylor *et al.*, 2000; Chatterji *et al.*, 2002; Porta *et al.*, 2003). Generally, provided the inserted peptide is less than 40 amino acids and has a pI below 9.0 (Porta *et al.*, 2003), the yields of modified particles are similar to those obtained with wild-type CPMV (up to 1 mg of particles per gram of infected leaf tissue). In each case, the chimeric virus particles present 60 copies of the inserted peptide on the virus surface, though preliminary experiments indicate that it will be possible to utilize more than one insertion site simultaneously. Where appropriate antisera are available, detection of the inserted epitope on the modified coat protein

subunits has proven to be straightforward (Usha *et al.*, 1993; Porta *et al.*, 1994, 1996).

Immunogenicity of CPMV chimeras

The first CPMV chimera and, indeed, the first modified plant virus particle to be assessed for the immunogenicity of a foreign peptide contained a 14–amino acid epitope (the NIm1A site) from human rhinovirus 14 (HRV-14). The modified particles proved capable of inducing specific antibodies against the insert when supplied parenterally to rabbits (Porta *et al.*, 1994). However, due to the nature of the expressed sequence, the antisera were nonneutralizing. Nonetheless, CPMV chimeras expressing this site have proven valuable in assessing the effect of mode of presentation of epitopes on their immunological structure. In particular, it allowed the effect of the proteolytic cleavage that occurs at or near the carboxy-terminus of the inserted sequence to be investigated (Lin *et al.*, 1996; Taylor *et al.*, 1999, 2000).

A number of CPMV-based chimeras have been subjected to detailed immunological analysis. The first to be analyzed in this regard was a chimera expressing a 22–amino acid epitope from gp41 (the "Kennedy epitope") of human immunodeficiency virus type 1 (CPMV-HIV/1; Porta *et al.*, 1994). Initial analysis of the immunogenicity of this chimera focused on the parenteral administration of purified particles to mice in the presence of alum adjuvant (McLain *et al.*, 1995). Sera obtained after boosting gave a strong ELISA response against the gp41 peptide. At a 1:100 dilution, antisera from all of the mice were found to be neutralizing against three strains of HIV-1, IIIB, RF, and SF2 (McLain *et al.*, 1995; 1996a). Further studies showed that neutralizing antibody production elicited by CPMV-HIV/1 did not have a narrow genetic restriction (McLain *et al.*, 1996b). An investigation into the effectiveness of five adjuvants at enhancing the immune response to CPMV-HIV/1 in mice showed that Quil A was the most effective of those tested (McInerney *et al.*, 1999). It was also the only one to stimulate an in vitro proliferative T-cell response. An additional, potentially significant finding from these experiments was that mice receiving chimeric particles in the absence of any adjuvant were also able to mount a secondary immune response. This indicates that the particles themselves can present antigens effectively to the immune system and may obviate the need to use adjuvants for at least some applications.

A curious feature of the immune response to CPMV-HIV/1 was that when the dose of particles was decreased from 100 μg to 1 μg there was a >230-fold decrease in the ELISA antibody titer while the neutralizing antibody titer dropped only twofold (McLain *et al.*, 1996a,b). The differential response of the ELISA and neutralizing antibody titers provided the first indication that mice make HIV-1-specific antibodies to two distinct epitopes present in the gp41 peptide, one neutralizing and one nonneutralizing. The presence of two such epitopes has subsequently been demonstrated (Buratti *et al.*, 1998). The nonneutralizing epitope is antigenically dominant, and deletion of this epitope results in a stronger neutralizing antibody response (Cleveland *et al.*, 2000a). The dissection of the "Kennedy epitope" through the use of CPMV-based chimeras has made a significant contribution to our understanding of the topology of gp41 from HIV-1 (Cleveland *et al.*, 2000b).

The key demonstration of the utility of plant virus–based vaccines, the ability to stimulate protective immunity, has been reported (Dalsgaard *et al.*, 1997) **(Table 59.1)**. This study involved a CPMV chimera (CPMV-PARVO1) that contained a 17–amino acid epitope from the N-terminal region of the VP2 capsid protein of canine parvovirus (CPV). This peptide is also found in VP2 of related parvoviruses, mink enteritis virus (MEV), and feline panleukopenia virus (FPV). CPMV-PARVO1 was administered subcutaneously to mink as a single dose of 100 μg or 1 mg with use of an alum/Quil A adjuvant, and 4 weeks' postvaccination the animals were challenged with MEV. Protection was afforded by either dose, and shedding of MEV was almost completely eliminated with the 1-mg dose. Subsequently, an ultraviolet light-inactivated form of CPMV-PARVO1 was shown to be capable of protecting dogs against a lethal challenge with CPV (Langeveld *et al.*, 2001). As in the case of the mink experiments, the chimera was mixed with an alum/Quil A adjuvant prior to inoculation, and a comparatively high dose (7.5 mg of purified particles per animal) was used. The levels of antibody response, protection, and virus shedding were similar to those obtained with the CPV peptide linked to keyhole limpet hemocyanin.

The work described above all concerned the properties of CPMV chimeras that express epitopes of viral origin. However, there have also been reports of immunological studies on chimeras expressing epitopes of bacterial origin. For example, the properties of a chimera expressing the 30–amino acid D2 domain of the fibronectin-binding protein (FnBP) from *Staphylococcus aureus* has been investigated (Brennan *et al.*, 1999b). The chimera was able to elicit antibodies in rats that completely inhibited the binding of fibronectin to immobilized FnBP and blocked the adherence of *S. aureus* to fibronectin. The construct was subsequently found to protect rats against endocarditis, and the serum from the rats protected mice against weight loss due to *S. aureus* bacteremia (Rennermalm *et al.*, 2001).

The construction of a chimera, CPMV-PAE5, which expressed a 34–amino acid sequence containing two epitopes (peptides 10 and 18) from the outer membrane (OM) protein F of *Pseudomonas aeruginosa* in tandem, has been described (Brennan *et al.*, (1999a). The antibodies induced in mice to this chimera, which were exclusively directed against the peptide 10 epitope, were able to recognize the F protein from all seven immunotypes of *P. aeruginosa*. Furthermore, immunized mice were protected against challenge by two different immunotypes of *P. aeruginosa* in a model of chronic pulmonary infection (Brennan *et al.*, 1999c; Table 59.1).

Tobacco mosaic virus

Construction of tobacco mosaic virus chimeras

Particles of tobacco mosaic virus (TMV) consist of a single molecule of genomic RNA (Fig. 59.1) encapsidated by 2130

Table 59.1. Protective Immunity Induced by the Administration of Peptides and Proteins Expressed in Plants with Use of Viral Vectors

Type of Virus Construct	Expressed Sequence	Species Protected	Immunization Route	Reference(s)
CPMV chimera	CPV VP2 epitope	Mink, dog	Parenteral	Dalsgaard *et al.* (1997); Langeveld *et al.* (2001)
CPMV chimera	*P. aeruginosa* OMF protein epitopes	Mouse	Parenteral	Brennan *et al.* (1999)
CPMV chimera	*S. aureus* D2 domain of FnBN	Rat	Parenteral	Rennermalm *et al.* (2001)
TMV chimera	MHV spike protein epitope	Mouse	Parenteral, nasal	Koo *et al.* (1999)
TMV chimera	*P. aeruginosa* OMF protein epitopes	Mouse	Parenteral	Stacek *et al.* (2000)
TMV/AlMV chimera	Rabies virus epitopes	Mouse	Parenteral, oral	Modelska *et al.* (1998)
TMV-expressed protein	FMDV VP1	Mouse	Parenteral	Wigdorovitz *et al.* (1999)
PPV-expressed protein	RHDV VP60	Rabbit	Parenteral	Fernandez-Fernandez *et al.* (2001)
PVX-expressed protein	HPV-16 E7	Mouse	Parenteral	Franconi *et al.* (2002)

copies of the 17.5-kDa coat protein arranged with helical symmetry. The first example of the presentation of foreign peptides on the TMV coat protein was reported in 1986 (Haynes *et al.*, 1986). However, in this case the modified coat protein bearing an eight–amino acid poliovirus epitope at its C-terminus was expressed in *Escherichia coli* rather than in plants. The production of modified TMV particles in plants was first attempted in 1990 (Takamatsu *et al.*, 1990). In these experiments, a sequence encoding Leu-enkephalin was fused to the C-terminus of the viral coat protein. However, the modified coat protein was not competent for virion assembly. This highlighted a problem with using TMV particles to express foreign peptides. The fact that TMV particles contain a large number of subunits, making the system potentially very attractive for peptide expression, is also a problem in that the subunits are very tightly packed, allowing little space on the virus surface for the expression of foreign sequences (Fig. 59.2).

To address the problem of steric hindrance, a TMV vector was developed that permitted the synthesis of both native and C-terminally modified versions of the coat protein from the same viral RNA (Hamamoto *et al.*, 1993). This was achieved by engineering a leaky termination codon at the C-terminus of the coat protein gene. This system produced particles in plants in which up to 5% of the coat protein subunits were modified at their C-termini and has been used to express epitopes from several animal pathogens (Sugiyama *et al.*, 1995; Turpen *et al.*, 1995). As with CPMV-based chimeras, the inserted peptides could be detected on the surface of assembled virions. Subsequently, by modifying the site of peptide insertion, TMV-based systems were developed in which all the coat protein subunits could be modified to express foreign peptides without abolishing virus

viability (Turpen *et al.*, 1995; Fitchen *et al.*, 1995; Beachy *et al.*, 1996). As a result, most TMV-based chimeras now contain inserts between amino acids 154 and 155, near but not at the C-terminus of the coat protein (Fig. 59.2). The size of inserts that can be tolerated even at this optimized position seems to be quite small, the largest reported to date being 23 amino acids in a chimera that grew substantially more slowly than wild-type TMV (Bendahmane *et al.*, 1999).

Immunogenicity of TMV chimeras
The first analysis of the immunogenicity of a TMV chimera involved a construct expressing 13 amino acids from the glycoprotein ZP3 from the murine zona pellucida (Fitchen *et al.*, 1995), a sequence previously shown to be capable of inducing antibody-mediated contraception. The modified virions were capable of eliciting antibodies in mice that bound to the zona pellucida, but the effectiveness of these antibodies in contraception could not be assessed.

Using the vector developed by Fitchen *et al.* (1995), Koo *et al.* (1999) expressed two sequences, of 10 and 15 amino acids, from the 5B19 epitope from the spike protein of the coronavirus, murine hepatitis virus (MHV). Counterintuitively, the construct harboring the 15–amino acid insert (TMV-5B19L) could be purified more readily than that with the smaller one (TMV-5B19), despite the latter sequence being entirely contained within the former. Mice immunized nasally (5B19L) or subcutaneously (5B19L or 5B19) with purified virions produced antibodies against the MHV epitope, and those with high antibody titers were protected from subsequent nasal challenge with the virus (Table 59.1).

In experiments similar to those reported previously with CPMV (Brennan, 1999a,c), Stacek *et al.* (2000) expressed

an epitope (peptide 9) from the OM protein F of *P. aeruginosa* on the surface of TMV (Stacek *et al.*, 2000). As found with the CPMV construct expressing different epitopes (peptides 10 and 18), antibodies induced in mice to the TMV chimera recognized the F protein from all seven immunotypes of *P. aeruginosa*, and immune mice were protected against challenge with *P. aeruginosa* (Table 59.1). In an attempt to develop a combined vaccine in which several epitopes from protein F of *P. aeruginosa* are presented simultaneously, mice were immunized with a mixture of the TMV chimera expressing peptide 9 and a chimeric influenza virus containing peptide 10 (Gilleland *et al.*, 2000). The mice produced antibodies against both epitopes and were protected against challenge at a level similar to that found when the individual components were used.

To overcome the limitation on the size of peptide that can be fused to the TMV coat protein, others have developed an approach that combined the use of TMV as vector with the known ability of the coat protein of alfalfa mosaic virus (AlMV) to tolerate foreign peptides at its N–terminus (Yusibov *et al.*, 1997). In this system, an appropriately modified version of the AlMV coat protein is expressed from an additional copy of the TMV coat protein subgenomic promoter. Using this approach, a 40–amino acid sequence containing epitopes from the glycoprotein and nucleoprotein of rabies virus and a 47–amino acid sequence from gp120 from HIV-1 were fused to the AlMV coat protein and the fusion protein expressed in *Nicotiana benthamiana*. In infected leaf tissue, the modified AlMV CP subunits assembled into ellipsoid particles that expressed multiple copies of the antigenic insert. When purified and injected into mice, these particles elicited the production of appropriate virus-neutralizing antibodies, even in the absence of adjuvant. Particles expressing the rabies virus epitopes were subsequently shown to protect mice against a normally lethal challenge with the virus when supplied either intraperitoneally or orally (Modelska *et al.*, 1998; Table 59.1).

In a related epitope/polypeptide approach, others have expressed a fusion protein consisting of a potentially neutralizing epitope from hepatitis C virus (HCV) linked to the C-terminus of the cholera toxin B subunit (CT-B) (Nemchimov *et al.*, 2000). Plants infected with the recombinant TMV produced functionally active pentameric CT-B presenting the inserted epitope. Nasal administration of crude plant material to mice elicited the production of antibodies against both the HCV epitope and CT-B.

Other plant viruses
Tomato bushy stunt virus
Tomato bushy stunt virus (TBSV) is a monopartite virus (Fig. 59.1), particles of which contain 180 copies of a single type of coat protein arranged with icosahedral symmetry. Sequences derived from gp120 of HIV-1 have been fused to the C-terminus of the coat protein, and the ability of the modified virus to infect *N. benthamiana* has been examined (Joelson *et al.*, 1997). When a sequence encoding 162 amino acids was expressed at this site, a large proportion of the inserted sequence was lost on serial passaging. By contrast, when a 13–amino acid sequence, corresponding to the V3 loop of gp41, was expressed at the same location, the construct was genetically stable and the inserted epitope could be detected immunologically. Though the modified virions stimulated only a weak response when injected into mice (Sjölander *et al.*, 1996), plates coated with particles could detect anti-V3 antibodies in HIV-positive individuals (Joelson *et al.*, 1997).

Plum pox virus
Plum pox virus (PPV) has flexuous rod-shaped particles consisting of more than 2000 copies of a single-coat protein encapsidating a single RNA molecule (Fig. 59.1). Though a detailed structure of PPV coat protein was not available, immunological analyses of related viruses suggested that both the N- and C-termini are surface-exposed. Furthermore, it had been shown that it was possible to fuse foreign sequences to the N-terminus of the coat protein of Johnsongrass mosaic virus (JGMV), a member of the same genus as PPV, without abolishing the ability of the coat protein molecules to assemble into viruslike particles in heterologous systems (Jagadish *et al.*, 1993). Making use of this information, one group fused a 15–amino acid epitope, equivalent to that used by Dalsgaard *et al.* (1997), from VP2 of CPV to a position near the N-terminus of the PPV coat protein, either as a single copy or as a tandem duplication (Fernandez-Fernandez *et al.*, 1998). Both constructs could be propagated in *N. clevelandii* and gave yields of virus particles similar to those obtained with wild-type PPV, and the inserted epitope could be detected on the virion surface. Antisera produced in either mice or rabbits with specificity for particles of either construct showed neutralizing activity in a monolayer-protection assay. The site of expression of peptides on the PPV coat protein has subsequently been refined (Fernandez-Fernandez *et al.*, 2002), raising the prospect that PPV may have general utility as an epitope-presentation system.

Potato virus X
Potato virus X (PVX) has filamentous particles consisting of approximately 1260 coat protein subunits encapsidating a single RNA molecule (Fig. 59.1). It has proven possible to express proteins at the surface–exposed N-terminus of either a proportion (Santa Cruz *et al.*, 1996) (see discussion of PVX in the section on Polypeptide Expression Systems) or all of the subunits (Marusic *et al.*, 2001). To assess whether peptides expressed in this way were immunogenic, one group fused a 38–amino acid sequence from the D2 domain of the fibronectin-binding protein (FnBP) from *Staphylococcus aureus* (Brennan *et al.*, 1999). The sequence was a C-terminally extended version of the 30–amino acid sequence from the epitope expressed on CPMV (see section on Immunogenicity of CPMV Chimeras). In the resulting chimeric particles it was estimated that about 10% of the subunits carried the inserted sequence. Though some of

the details varied, the immunogenicity of the PVX construct was generally similar to that of the equivalent CPMV one. Using a related approach, one group expressed a highly conserved hexapeptide epitope from gp41 of HIV-1 on all the PVX subunits (Marusic *et al.*, 2001). Mice immunized either intraperitoneally or nasally with the chimeric particles produced high levels of HIV-1-specific IgG and IgA antibodies. The potential human response to the chimera was assessed using immunodeficient mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID mice). These mice made human primary antibody responses to the gp41 peptide, and the serum exerted an anti-HIV-1-neutralizing activity.

Alfalfa mosaic virus
Recently, an attempt to express the rabies epitopes described in the section on Immunogenicity of TMV Chimeras on the surface of AlMV particles using an AlMV rather than a TMV vector was reported (Fleysch *et al.*, 2001). The potential advantage of using AlMV itself rather than TMV is that the former can infect edible plants such as soybean. Though the initial experiments appeared promising, it has not been possible to reproduce them (Brodzik *et al.*, 2002).

POLYPEPTIDE EXPRESSION SYSTEMS

A number of RNA viruses, particularly but not exclusively those which produce rod-shaped or filamentous particles, have been adapted to act as vectors for the production of free foreign proteins in plants. The preference for these types of virus over those with isometric particles is mainly due to the absence of any absolute limit on the size of RNA that can be packaged in the particles.

Tobacco mosaic virus
The first virus to be used to express a whole protein (chloramphenicol acetyl transferase, or CAT) was TMV (Takamatsu *et al.*, 1987; Dawson *et al.*, 1989). Subsequently, TMV vectors were developed in which expression of an inserted sequence is driven by an additional copy of the coat protein subgenomic promoter (Donson *et al.*, 1991), with genetic stability being improved by the use of promoters from heterologous strains. These have been used to express high levels (up to 2% of soluble proteins) of several valuable proteins in plants. These include a eukaryotic ribosome-inactivating protein (RIP), α-trichosanthin (Kumagai *et al.*, 1993), and single-chain (ScFvs; McCormick *et al.*, 1999) or full-length monoclonal antibodies (Verch *et al.*, 1998). Furthermore, it has proven possible to synthesize glycosylated proteins with TMV vectors (Kumagai *et al.*, 2000; Dirnberger *et al.*, 2001).

There are a number of examples in which the TMV vector system has been used to produce immunogenic proteins. One group (Wigdorovitz *et al.*, 1999) expressed VP1 from FMDV in *N. benthamiana* using the vector developed by others (Donson *et al.*, 1991). This group detected significant

quantities (50–150 µg per gram of leaf, fresh weight) of VP1 in leaves and used leaf extracts to inoculate mice intraperitoneally in the presence of Freund's complete adjuvant. All mice immunized in this manner developed significant antibody titers directed to a VP1 epitope. When challenged with FMDV, all immunized mice were protected in two separate experiments (Table 59.1). This was the first example in which a whole protein, rather than a peptide fusion, expressed in plants using a viral vector was shown to be capable of conferring protective immunity. Another group expressed a major birch pollen antigen (Betv1) in *N. benthamiana* and showed that the B-cell epitopes from natural Betv1 were preserved in the plant-expressed protein (Krebitz *et al.*, 2000). Mice immunized with crude leaf extracts from *N. benthamiana* expressing Betv1 generated immunological responses comparable to those induced by the protein expressed in *E. coli* or extracted from birch pollen.

Potato virus X
Potato virus X (PVX) has been used to express polypeptide immunogens in two different formats. The first uses duplicated subgenomic promoters (Chapman *et al.*, 1992) and is similar to that developed with TMV (see discussion on TMV in section on Polypeptide Expression Systems; Fig. 59.1). Such vectors have proven very useful in expression studies with a number of proteins such as ScFv antibodies (Hendy *et al.*, 1999; Franconi *et al.*, 1999; Ziegler *et al.*, 2000). In terms of immunogens, there are only two examples. One group expressed the major capsid protein, VP6, from a murine rotavirus and showed that although the protein retained its ability to form trimers, it tended to assemble into paracrystalline sheets and tubes rather than viruslike particles (VLPs) (O'Brien *et al.*, 2000). Another group used PVX to express the E7 protein human papillomavirus 16 (HPV-16), a virus implicated in the induction of cervical cancer, in *N. benthamiana* (Franconi *et al.*, 2002). Mice immunized with foliar extracts containing the E7 protein in the presence of Quil A as an adjuvant developed both antibody and cell-mediated immune responses. The isotypic profile of the IgG antibodies indicated that both Th1 and Th2 responses were present, and 40% of mice remained tumor-free after challenge with an E7-expressing tumor cell line. The tumors that did develop in mice vaccinated with the plant-expressed E7 were considerably smaller in volume than those that developed in untreated mice (Table 59.1). The results obtained in this study were particularly significant, as previous attempts to produce large amounts of unfused E7 in other expression systems had been unsuccessful.

The second type of PVX polypeptide expression system involves the fusion of the foreign protein to the N-terminus of the coat protein gene via the 2A catalytic peptide from FMDV. The 2A sequence promotes cotranslational cleavage between the foreign gene insert and the CP, although this is not 100% efficient, resulting in some CP subunits still bearing the inserted protein. These fusion proteins were found to retain their ability to be incorporated into virus capsids, resulting in particles that display the inserted

polypeptide. By means of this approach it is possible, with the use of the same construct, to produce a protein of interest in both a free (unfused) state where cleavage by 2A has occurred and as a CP fusion where it is incorporated in PVX particles. The functionality of a foreign protein when incorporated into virions was demonstrated by the observation that an ScFv expressed as a CP fusion could still bind to its antigen, the herbicide Diuron (Smolenska et al., 1998). When this system was used to express the rotavirus VP6 sequence, the uncleaved VP6-2A-CP assembled into PVX virions while the VP6-2A cleavage product formed typical VP6 VLPs (O'Brien et al., 2000).

Plum pox virus

The PPV genomic RNA contains a single ORF that encodes a multifunctional polyprotein (Fig. 59.1). This is self-processed by proteinase domains within it to produce the mature viral proteins. To develop PPV as a polypeptide expression system, sequences encoding foreign proteins, initially marker genes, were inserted such that the free polypeptide would be released through the action of the VPg-proteinase (Fig. 59.1). This was achieved by flanking the inserted sequence with the appropriate proteinase recognition sites (Guo et al., 1998). Subsequently, a PPV vector in which the foreign sequence was inserted between the polymerase (Pol) and coat protein (CP) genes (Fig. 59.1) was used to express the VP60 structural protein from the calicivirus, rabbit hemorrhagic disease virus (RHDV) in *N. clevelandii* (Fernandez-Fernandez et al., 2001). Inoculation with a crude preparation from infected leaf tissue in the presence of adjuvant fully protected rabbits against subsequent challenge with a lethal dose of RHDV (Table 59.1). Taking into account the dose required to confer immunity and the time taken to grow the PPV-infected plants, Fernandez-Fernandez et al. (2001) estimated that 1 m² of greenhouse space and 21 days would be sufficient to obtain enough material to protect 50 rabbits against RHDV.

Tomato bushy stunt virus

To overcome the potential limitation on the size of insertion that could be tolerated in the isometric TBSV particles, one group exploited the fact that the TBSV coat protein is not essential for infectivity and produced constructs in which most of the region encoding the coat protein was replaced with marker genes (Fig. 59.1) (Scholtof et al., 1993). A refined version of the vector was subsequently produced in which the coat protein gene was replaced with a polylinker (Scholtof, 1999). This, coupled with improvements to the infection process, permitted the facile expression of heterologous sequences in the inoculated leaves of plants. The approach has been used to express the nucleocapsid protein p24 from HIV-1 as a fusion with the 5′ terminal portion of the CP gene (Zhang et al., 2000). The modified TBSV RNA was capable of replicating in both protoplasts and in the inoculated leaves of whole plants. Accumulation of the CP-p24 fusion protein could be detected in inoculated leaves but, as yet,

there has been no report concerning the immunological properties of the plant-expressed protein.

MUCOSAL IMMUNIZATION

One of the potential advantages of using plants to produce vaccines is that it may be possible to supply the immunological material to mucosal surfaces without a high degree of purification. However, to demonstrate the immunogenicity of plant-expressed material, initial investigations involved parenteral administration in the presence of adjuvant (Table 59.1). However, parenteral immunization does not generally elicit a mucosal immune response, and there have been a number of recent studies aimed at investigating the efficacy of supplying material by other routes.

To investigate whether purified particles of CPMV chimeras can elicit a mucosal immune response, one group administered CPMV-HIV/1 (see section on Immunogenicity of CPMV Chimeras) particles either nasally or orally in the presence of cholera toxin as an adjuvant (Durrani et al., 1998). All the mice immunized nasally produced anti-HIV IgA in feces as well as serum IgG antibodies. Oral immunization was considerably less effective, with only serum antibody being stimulated in a minority of mice. Similar results were obtained when mice were immunized nasally or orally with a CPMV chimera expressing a 30–amino acid sequence from the *S. aureus* FnBP (Brennan et al., 1999d). Detailed characterization of the immune responses in mice immunized nasally with CPMV chimeras expressing epitopes from CPV provided further support to the idea that it will be possible to stimulate mucosal immunity by this route (Nicholas et al., 2002).

Evidence that it may, in fact, be possible to administer CPMV-based chimeras orally was provided by studies on the related virus, cowpea severe mosaic virus (CPSMV). When purified virus particles or crude extracts of CPSMV-infected cowpea leaves were used to immunize mice orally, anti-CPSMV IgG and IgA but not IgE antibodies were produced systemically (Florindo et al., 2002). Significantly, no antibodies were produced in response to the leaf proteins from either healthy or CPSMV-infected cowpeas, and no pathogenic effects were noted in any of the mice. Though these results were obtained with a wild-type virus rather than a chimera, they indicate that purification of virus particles may not be required prior to oral immunization.

The most thorough study to date on the effectiveness of different modes of immunization on protective immunity was carried out by a group using chimeric AlMV viruslike particles carrying rabies virus epitopes (see section on Immunogenicity of TMV Chimeras) (Modelska et al., 1998). The immunogen was supplied to mice in three different ways: intraperitoneally, by gastric intubation, or by feeding on virus-infected leaves. The first two routes involved supplying purified virus particles, while the third used spinach leaves containing the recombinant protein. Though less effective in affording protection than the intraperitoneal

route, both forms of oral administration stimulated IgG and IgA antibody synthesis and ameliorated symptoms of a subsequent challenge with an attenuated strain of rabies virus. In similar experiments, others compared the efficacy of purified particles of TMV chimeras expressing an epitope from the spike protein of MHV (see section on Immunogenicity of TMV Chimeras) when supplied to mice either subcutaneously in the presence of adjuvant or nasally (Koo *et al.*, 1999). Nasal administration induced the production of epitope-specific IgG and IgA antibodies, while subcutaneous administration led to the production of only IgG antibodies. In both cases, mice were protected from subsequent challenge with MHV, and there appeared to be some correlation between the degree of protection obtained and the IgA antibody titers.

REGULATORY ISSUES

There are two types of regulatory issues that will need to be addressed before plant-based vaccines using viral vectors can be used. The first type concerns the equivalence between plant-expressed proteins and those produced by more conventional means. These concerns apply generically to proteins expressed in plants whether they are produced transgenically, transiently, or through the use of viral vectors. The problems in demonstrating equivalence have been specifically reviewed elsewhere (Miele, 1997) and will not be further discussed here.

The second type of issue is specifically related to the use of virus-based vectors for foreign gene expression. These principally concern the genetic stability of the expressed sequences and the possibility of spread of the modified viruses in the environment. RNA viruses are particularly prone to the accumulation of mutations during multiple rounds of replication, as RNA-dependent RNA polymerases lack proofreading functions. It was anticipated that this could be a particular problem when expressing heterologous sequences, since their presence, far from conferring a selective advantage on the virus, tends to reduce the rate of virus replication. Thus, the inserted sequence tends to be subjected to both genetic drift and deletion. Indeed, when RNA viruses were first mooted as potential vectors, there was some debate as to whether they would be of any practical use for the expression of foreign proteins (van Vloten-Doting *et al.*, 1985; Siegel, 1985). However, studies on the stability of both constructs containing either whole genes or expressing peptides indicate that these problems will be manageable, provided excessive passaging is not undertaken (Kearney *et al.*, 1993, 1999; Porta *et al.*, 1994).

The second concern involves the potential of modified viruses to spread in the environment. This could lead to the expression of immunologically active material in unwanted locations, and the presence of the foreign sequence could conceivably alter the host range and/or the transmissibility of the modified virus. Complete elimination of the ability of modified viruses to infect healthy host plants is clearly unde-

sirable, as it is this property that makes viral vectors attractive in the first place. However, limitation of the ability to spread would be an advantage. At present this is most commonly achieved by growing infected plants under physically contained conditions, but this may not always be possible if large quantities of material are to be produced. In a pilot experiment to assess the potential spread of recombinant viruses in a field situation, adjacent rows of tobacco in a field were spray-inoculated with two different recombinant TMV constructs (Pogue *et al.*, 2002). No cross-contamination between plants was found, despite the rows being only 18 inches apart. In addition, the fact that recombinants are at a disadvantage in terms of replication rate when compared with the wild-type virus leads to the deletion of the inserted sequence after multiple rounds of multiplication (Guo *et al.*, 1998; Kearney *et al.*, 1999; Rabindran and Dawson, 2001). This is likely to effectively prevent the widespread unwanted expression of foreign protein in the environment during propagation.

To address the problem of potential virus spread after material has been purified and/or administered to animals, one group carried out ultraviolet inactivation experiments on CPMV-PARVO1 (see section on Immunogenicity of CPMV Chimeras) (Langeveld *et al.*, 2001). This group showed that it was possible to completely abolish the infectivity of chimera on plants without affecting either the structural integrity of the particles or their ability to stimulate protective immunity. Since ultraviolet irradiation is a U.S. Food and Drug Administration–approved method for virus inactivation, this method may be a generally useful pretreatment for purified preparations of chimeric virus particles.

The possibility that a modified virus may have its host range or transmission ability altered has been raised in the case of chimeric virus particles. Though there is no evidence that host range is determined directly by the sequence of the viral coat protein, transmission is known to be affected. Thus, it is conceivable that expression of a foreign peptide on the surface of virus particles may, by chance, change the ability of a virus to spread in the environment. To investigate this possibility, one group examined the host ranges and transmission characteristics of two CPMV chimeras, expressing epitopes from HRV-14 and HIV-1 (Porta *et al.*, 2003). The host ranges of the chimeras were identical to that of wild-type CPMV, but they had a reduced ability to be transmitted by beetles, the natural insect vectors. Furthermore, the chimeras had not gained an ability to be transmitted by aphids or through seed. Though these experiments were inevitably somewhat limited in scope, they have provided further support to the idea that incorporation of foreign peptides on a virus surface if anything reduces the ability of the virus to spread in the environment.

SUMMARY AND FUTURE PROSPECTS

The past few years have seen significant progress in the use of plant virus vectors for the production of immunogens in plants. Particularly encouraging is the fact that it is now clear that

peptides or proteins expressed in this manner can confer protective immunity against a number of diseases (Table 59.1), in some cases in the target animals (Dalsgaard *et al.*, 1997; Langeveld *et al.*, 2001; Fernandez-Fernandez *et al.*, 2001). In most of these cases, immunity was stimulated by parenteral immunization, but there are encouraging signs that mucosal immunization may be possible (Modelska *et al.*, 1998; Koo *et al.*, 1999; Durrani *et al.*, 1999). This raises the prospect that it may be possible to confer protective immunity by simply feeding with plant material infected with an appropriate virus construct. To achieve this, it will be necessary not only to express peptides or proteins that can stimulate mucosal immunity but also to express the material in edible plants. In this regard, the continued development of vectors that can infect edible plants such as those based on CPMV (Gopinath *et al.*, 2000), clover yellow vein virus (ClYVV; Masuta *et al.*, 2000), and pea early browning virus (PEBV; MacFarlane and Popovich, 2000), all of which infect legumes; wheat streak mosaic virus (WSMV; Choi *et al.*, 2000), which infects cereals; and zucchini yellow mosaic virus (ZYMV; Arazi *et al.*, 2001), which infects cucurbits, is likely to play a prominent role. In addition, the development of combined transgene/virus complementation systems, such as that described by others (Sanchez-Navarro *et al.*, 2001; Mori *et al.*, 2001), may allow the use of defective viral replicons for the expression of foreign sequences, thereby reducing the risk of environmental spread.

Even if direct feeding of plant material is shown to be efficacious, there will still be need to purify, at least partially, proteins and chimeric particles for certain applications. Though sufficient material for initial characterization can be obtained by laboratory-scale extractions, a substantial scale-up of procedures will be necessary for its widescale use. One group reported the results of experiments on the large-scale growth and purification of a TMV chimera expressing a 12–amino acid malarial peptide (Pogue *et al.*, 2002). The results indicate that although growth under field conditions gives a lower yield per gram (fresh weight) of tissue than growth in a greenhouse or growth chamber, in excess of 1 kg per acre (planted area) of purified particles could be obtained. It was shown that large quantities (up to 10 mg per gram, fresh weight) of wild-type CPMV could be extracted from fresh or frozen cowpea leaves by methods suitable for large-scale application (Nichols *et al.*, 2002). The development of methods for the industrial-scale production and/or purification of plant-derived vaccines will make an important contribution to the practical use of such material.

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