Special Feature

Use of viral vectors for vaccine production in plants

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Summary The small size of plant viral genomes, the ease with which they can be manipulated, and the simplicity of the infection process is making the viral vectors an attractive alternative to the transgenic systems for the expression of foreign proteins in plants. One use of these virus expression systems is for vaccine production. There are two basic types of viral system that have been developed for the production of immunogenic peptides and proteins in plants: epitope presentation and polypeptide expression systems. In this review, we discuss advances made in this field.

Key words: epitope presentation, plant virus, polypeptide expression, viral vector.

Introduction

The use of plants for vaccine production is emerging as an attractive alternative to traditional production systems. Plant expression systems have several advantages such as the absence of risk of contamination with animal pathogens, potentially low production costs, and the possibility of very large-scale production. They also offer the option of producing edible vaccines.

The classical system for expressing foreign proteins in plants is stable genetic transformation. This approach involves integration of the gene of interest into the plant genome, and it has been used successfully to express a number of immunologically active proteins. An alternative to stable genetic transformation is the use of plant virus-based vectors to achieve expression. The main advantages of using the latter strategy are that viral genomes are small and easy to manipulate, infection of plants with modified viruses is simpler and quicker than the regeneration of stably transformed plants, and the sequence inserted into a virus vector will be highly amplified. However, there are some disadvantages: 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. Despite these difficulties, different plant virus vectors have been used for protein expression. In the present paper, we review the development of virus-based vectors as a potential source of novel vaccines in plants.

Types of viral expression system

The first attempts to develop plant virus vectors involved viruses with DNA genomes. Unfortunately, the complexity of the replication cycles made these viruses unsuitable for the expression of high levels of foreign proteins in plants. To gain

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information about these DNA virus-based vectors, the reader is referred to Porta and Lomonossoff.[1,2]

The vast majority of plant viruses have genomes that consist of one or more molecules of positive-sense RNA. These viruses can grow in a wide range of hosts, and some can reach extremely high titres. The ability of the genomes of positive-sense RNA plant viruses to be directly translated on entering a plant cell has made this class of virus a very attractive choice to express recombinant proteins in infected plants.[3] With the development of methods that permit the manipulation of infectious cDNA clones of positive-strand RNA viruses,[4] the exploitation of these viruses as vectors for foreign protein expression began. Since then, members of several virus families have been developed as useful vectors (Fig. 1). The first strategy used for developing virus vectors was to replace existing nonessential viral genes with foreign genes. However, this replacement strategy has limitations, due to adverse consequences associated with gene deletion. A more successful approach has been to insert the foreign gene as an addition to the virus genome, avoiding the deletion of any viral genes. For reviews on the development of RNA virus-based vectors, the reader is referred to Scholthof *et al*.[5] and Porta and Lomonossoff.[2,6]

Two basic types of expression system based on RNA plant viruses have been developed for the production of immunogenic peptides and proteins in plants. In the first type, termed epitope presentation systems, the viral vector is designed so that short antigenic peptides fused to the coat protein (CP) are displayed on the surface of assembled viral particles. These modified virions or chimeras are attractive as potential novel vaccines, because the modified particles can be readily purified and the presentation of multiple copies of an antigenic peptide on the surface of a macromolecular assembly can significantly increase its immunogenicity.[7] The second type, often referred to as polypeptide expression systems, express a whole unfused recombinant protein that accumulates within the plant.

Epitope presentation systems

In this expression strategy, the sites of insertion are chosen so that the peptide is displayed on the surface of the virus
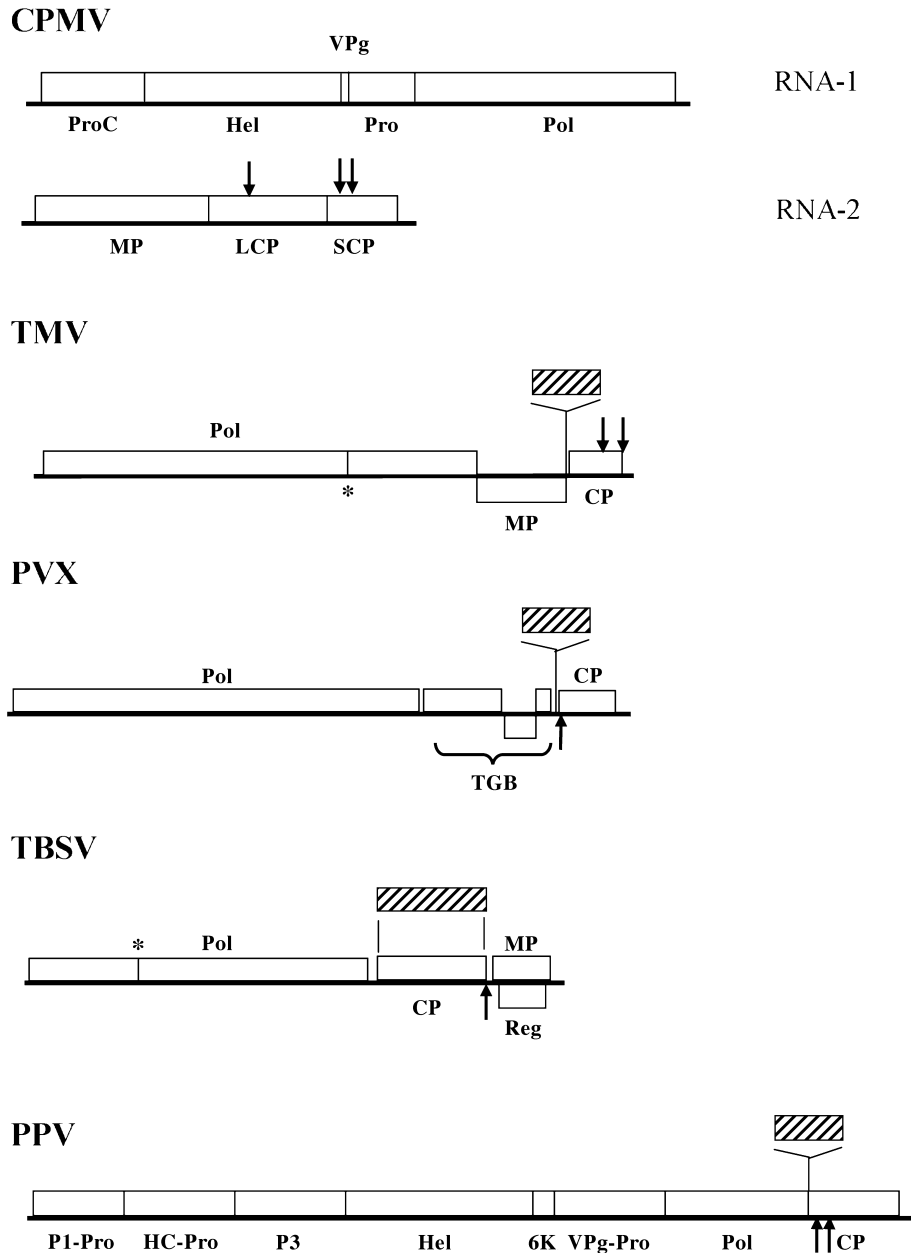
Figure 1 Genome organization of viruses used to express heterologous peptides and proteins in plants. Positions where epitopes have been inserted into the coat proteins of the various viruses are shown by black arrows. The positions where foreign proteins (shown hatched) have been inserted into the viral genomes are also indicated. The functions of various virus genes are shown as: 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; VPg-Pro, VPg-Proteinase. The asterisk (*) represents a leaky termination codon. CPMV, cowpea mosaic virus; PPV, plum pox virus; PVX, potato virus X; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus.

particles without interfering with the ability of the modified CP to assemble. For this reason, this system has mainly been developed for viruses where there is some information available about the topology of the CP in the assembled virions. The viral systems discussed below are the ones that are currently used for epitope presentation, but it is likely that this list will expand as structural information about more viruses becomes available.

Cowpea mosaic virus

Cowpea mosaic virus (CPMV) was the first plant virus to be developed as an epitope presentation system.[1,8,9] CPMV is a bipartite RNA virus (Fig. 1), particles of which are composed of 60 copies of each of large (L) and small (S) CP arranged with icosahedral symmetry. Given the state of knowledge of the structure of its particles, the availability of infectious cDNA clones and the potential productivity of the system, CPMV was an attractive candidate for development as an epitope presentation system. Analysis of the 3-D structure of CPMV identified exposed loops on the virus surface that would potentially be suitable sites for the insertion of foreign sequences.[10] In most cases, the foreign sequence has been inserted into the most exposed loop on the virus surface, the βB-βC loop of the S protein.[8,9] However, other sites, such as the βE-αB loop of the L protein and the βC′-βC″ loop of the S protein, have also been used successfully.[11–14] Generally, provided the inserted peptide has fewer than 40 amino acids and has a pI below 9.0,[14] the yields of modified particles are similar to those obtained with wild-type CPMV.

A number of CPMV-based chimeras have been subjected to detailed immunological analysis. Some of them, after being

purified and injected into experimental animals, have been shown to raise antibodies against the inserted peptide. These antibodies are able to bind to, and in several cases neutralize, the pathogen from which the peptide was derived. The reader is referred to Lomonossoff and Hamilton,[15] where the results of these immunological studies have been reviewed. The ability of modified plant virus particles to stimulate protective immunity, the ultimate goal of vaccine development, was first reported in 1997.[16] 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. This peptide is also found in VP2 of related canine parvoviruses (CPV), mink enteritis virus (MEV), and feline panleukopenia virus (FPV). This CPMV-PARVO1 chimera in an active and a UV light-inactivated form were shown to be capable of protecting mink and dogs against MEV and CPV, respectively.[16,17] CPMV-based chimeras expressing epitopes of a bacterial origin were subsequently shown to also be able to confer protective immunity[18,19] (Table 1).

Tobacco mosaic virus

Tobacco mosaic virus (TMV) particles consist of a single molecule of genomic RNA encapsidated by 2130 copies of a single type of CP arranged with helical symmetry. 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. Attempts to develop TMV as an epitope presentation system initially resulted in the production of CP subunits that were unable to assemble into virus particles. By engineering a leaky termination codon at the C-terminus of the CP gene, a TMV vector was developed that permitted the synthesis of both native and recombinant forms of the CP from the same viral RNA.[20] As with CPMV-based chimeras, the inserted peptides could be detected on the surface of assembled virions, being used to express epitopes of several animal pathogens.[21,22] Subsequently, by modifying the site of peptide insertion between amino acids 154 and 155, near but not at the C-terminus of the CP, it has been possible to develop a TMV-based vector in which all of the CP subunits could be modified to express foreign peptides abolishing virus viability.[23] 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.[24]

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[23] (Table 1). Using the vector developed by Fitchen *et al.*,[23] Koo *et al.*[25] were able to produce chimeric TMV particles presenting an epitope from mouse hepatitis virus (MHV). Mice immunized with purified virions produced antibodies against the MHV epitope, and those with high antibody titres were protected against challenge with the virus. Recently, using the same strategy, two epitopes from foot and mouth disease virus (FMDV) were expressed on TMV.[26] As reported previously with CPMV,[18] expression of an epitope from the OM protein F of *Pseudomonas aeruginosa* on the surface of TMV[27] has been shown to confer protective immunity against *P. aeruginosa*. In the same way, a mixture of a TMV chimera and a chimeric influenza virus presenting two different epitopes from protein F of *P. aeruginosa* conferred immunity in mice.[28]

To overcome the limitation on the size of peptide that can be fused to the TMV CP, researchers have developed a system where an appropriately modified version of the alfalfa mosaic virus (AlMV) CP is expressed from an additional copy of the TMV CP subgenomic promoter. Using this approach, a 40 amino acid sequence containing epitopes from rabies virus and a 47 amino acid sequence from HIV-1 were fused to the AlMV CP.[29] Both types of particle elicited the production of appropriate virus-neutralizing antibodies; those displaying the rabies virus epitopes were able to protect mice against a normally lethal challenge with the virus.[30]

Using a similar approach, a fusion protein consisting of a potentially neutralizing epitope from hepatitis C virus linked to the C-terminus of the cholera toxin B subunit has been expressed from TMV.[31]

Tomato bushy stunt virus

Tomato bushy stunt virus (TBSV) is a monopartite virus, particles of which contain 180 copies of a single type of CP arranged with icosahedral symmetry. Sequences derived from gp120 of HIV-1 have been fused to the C-terminus of the CP. A 13 amino acid epitope sequence, corresponding to the V3 loop, could be detected immunologically.[32]

Plum pox virus

Plum pox virus (PPV) has flexuous rod-shaped particles consisting of more than 2000 copies of a single CP encapsidating a single RNA molecule. Although a detailed structure of PPV CP was not available, immunological analyses of related viruses suggested that both the N- and C-termini are exposed to the surface. In addition, the demonstration that it is possible to fuse foreign sequences to the N-terminus of the heterologously expressed CP of Johnsongrass mosaic virus (JGMV),[33] a member of the same genus as PPV, without abolishing particle formation, has encouraged other groups to use a similar strategy *in planta*. The fusion of a 15 amino acid epitope, equivalent to that used by Dalsgaard *et al.*,[16] from VP2 of CPV to a position near the N-terminus of PPV CP, either as a single copy or as a tandem duplication,[34] gave yields of virus particles similar to those obtained with wild-type PPV. The site of expression of peptides on the PPV CP has subsequently been refined,[35] raising the prospect that PPV may have general use as an epitope presentation system.

Potato virus X

Potato virus X (PVX) has filamentous particles consisting of approximately 1260 CP subunits encapsidating a single RNA molecule. It has proven possible to express proteins at the surface-exposed N-terminus of either a proportion[36] or all of the subunits.[37]

Table 1 Immunogenic proteins reported using the viral epitope presentation system in plants

Epitope presentation system	Expressed epitope	Plant used for infection	Species protected	Immunological properties	Reference
CPMV	VP2 capsid protein of canine parvovirus (CPV)	*Vigna unguiculata*	Mink	Parenteral injection protected mink against challenge with mink enteritis virus (MEV)	16
CPMV	Canine parvovirus VP2	*Vigna unguiculata*	Dog	UV light-inactivated form of the chimera protected dogs against challenge with canine parvovirus (CPV)	17
CPMV	*Pseudomonas aeruginosa* outer membrane (OM) protein F	*Vigna unguiculata*	Mouse	Parenteral injection protected mice against challenge with *P. aeruginosa*	18
CPMV	*Staphylococcus aureus* D2 domain of fibronectin-binding protein (FnBP)	*Vigna unguiculata*	Rat	Parenteral application protected rats against endocarditis	19
TMV	Glycoprotein ZP3 from the murine zona pellucida	*Nicotiana tabacum*	Mouse	Modified virions were capable of eliciting antibodies in parenterally immunized mice	23
TMV	Mouse hepatitis virus (MHV)	*Nicotiana tabacum*	Mouse	Mice parenterally and intranasally immunized were protected against challenge with MHV	25
TMV	Foot and mouth disease virus (FMDV)	*Nicotiana tabacum*	Guinea pig	Oral immunization was less effective than the parenteral injection against FMDV challenge	26
TMV	*Pseudomonas aeruginosa* outer membrane (OM) protein F	*Nicotiana tabacum*	Mouse	Parenterally immunized mice were protected against challenge with *P. aeruginosa*	27
TMV	*Pseudomonas aeruginosa* outer membrane (OM) protein F	*Nicotiana tabacum*	Mouse	Mice immunized with a mixture of a TMV chimera and a chimeric influenza virus, parenterally and nasally administered, respectively, produced antibodies against the two different epitopes and were protected against challenge with *P. aeruginosa*	28
TMV/AIMV	Rabies virus HIV-1	*Nicotiana benthamiana* *Spinacia oleracea*	Mouse	Both epitopes produced virus-neutralizing antibodies, being the rabies ones able to protect mice when supplied either intraperitoneally or orally	29,30
TMV	Hepatitis C linked to the C-terminus of the cholera toxin B subunit gp120 of HIV-1	*Nicotiana benthamiana*	Mouse	Nasal administration of crude plant material elicited the production of antibodies against both epitopes	31
TBSV	gp120 of HIV-1	*Nicotiana benthamiana*	Mouse	Parenteral immunization only gave a relatively low synthetic peptide specific primary antibodies	32
PPV	VP2 capsid protein of canine parvovirus (CPV)	*Nicotiana clevelandii*	Mouse Rabbit	Parenteral immunization showed the presence of neutralizing antibodies against both CPV and PPV	34
PVX	gp41 of HIV-1	*Nicotiana benthamiana*	Mouse	Mice immunized intraperitoneally or nasally produced high levels of HIV-1 IgG and IgA antibodies; immunodeficient mice reconstituted with human peripheral lymphocytes made human primary neutralizing antibodies	37
AIMV	Respiratory syncytial virus (RSV) G protein	P12 transgenic *Nicotiana tabacum*	Monkey	Parenteral injection of recombinant particles generated T- and B-cell responses; *in vitro* human dendritic cells incubated with the chimera generated strong T-cell response	38

AIMV, alfalfa mosaic virus; CPMV, cowpea mosaic virus; PPV, plum pox virus; PVX, potato virus X; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus.

Alfalfa mosaic virus

It has recently been reported that a 21 amino acid peptide of the respiratory syncytial virus (RSV) G protein fused with the AlMV CP generated strong T-cell responses in human dendritic cells and both T- and B-cell responses in non-human primates to the incorporated RSV.[38]

Polypeptide expression systems

This type of viral expression system 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. Although purification of the expressed protein may be necessary or desirable, this type of system could be suitable for the production of immunogens that can be supplied orally by direct feeding of plant material to animals. A number of RNA viruses – mainly those without the limitation on the size of RNA that can be packaged in the particles – have been used. Among the main ones investigated are TMV, PVX, PPV, TBSV and, to a lesser extent, CPMV. A brief account on the development of viral vectors for the expression of foreign proteins using the polypeptide expression system is given below, and examples where immunogenic proteins have been produced are further illustrated in Table 2.

Tobacco mosaic virus

An early phase in the development of viral vectors such as those based on TMV was the expression of marker genes such as chloramphenicol acetyl transferase (CAT).[39,40] Further work included the expression of an inserted sequence under the control of an additional copy of the CP subgenomic promoter,[41] with genetic stability being improved by the use of promoters from heterologous strains. This resulted in high levels (up to 2% of soluble proteins) of several valuable proteins, including the eukaryotic ribosome-inactivating protein (RIP), α-trichosanthin,[42] single-chain antibodies (ScFv),[43] or full-length monoclonal antibodies.[44] Furthermore, it has proven possible to synthesize glycosylated proteins with TMV vectors.[45,46] There are a number of examples in which the TMV vector system has been used to express immunogenic proteins (Table 2). These include VP1 from FMDV,[47] a tumour-derived scFv epitope,[43] a major birch pollen antigen (Betv1),[48] and the cytosolic form of the bovine herpes virus gD protein.[49]

Potato virus X

The development of PVX-based viral vectors has been carried out in two different formats. The first uses duplicated subgenomic promoters[50] similar to those used for TMV. This strategy has been used to express a number of proteins such as ScFv antibodies,[51,52] the major capsid protein (VP6) from a murine rotavirus,[53] and the E7 protein of human papillomavirus 16.[54] The second type involves the fusion of the foreign protein to the N-terminus of the CP 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 displayed the inserted polypeptide. By means of this approach it is possible, using 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. A ScFv expressed as a CP fusion was found to be functional when incorporated into virions.[55] When this system was used to express the rotavirus VP6 sequence, the uncleaved VP6-2A-CP was incorporated into PVX virions while the VP6-2A cleavage product formed typical VP6 virus-like particles (VLP).[53]

Plum pox virus

Efforts in using the PPV-based viral vectors have concentrated on inserting the foreign sequence into the single open reading frame (ORF) that encodes a multifunctional polyprotein that is self-processed by proteinase domains within it to produce the mature viral proteins. Initial experiments involved inserting marker genes in such a manner that the free polypeptide would be released through the action of the VPg-proteinase (Fig. 1). This was achieved by flanking the inserted sequence with the appropriate proteinase recognition sites.[56] Subsequently, a PPV vector in which the foreign sequence was inserted between the polymerase (Pol) and CP genes (Fig. 1) was used to express the VP60 structural protein from rabbit haemorrhagic disease virus (RHDV).[57] Immunized rabbits were protected against subsequent challenge with a lethal dose of RHDV.

Tomato bushy stunt virus

The use of TBSV-based vectors has limitations on the size of insertion that the isometric particles can tolerate. To overcome this, one group exploited the fact that the TBSV CP is not essential for infectivity, and they produced constructs in which most of the region encoding the CP was replaced with marker genes.[58] A refined version of the vector was subsequently produced in which the CP gene was replaced with a polylinker.[59] This, coupled with improvements to the infection process, permitted the facile expression of heterologous sequences in the inoculated leaves of plants. This 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,[60] but there is no report as yet concerning the immunological properties of the plant-expressed protein.

Cowpea mosaic virus

Regarding CPMV-based vectors, efforts began with the expression of the green fluorescent protein (GFP) as a cytosolic form. CPMV RNA-2 was engineered to accommodate the FMDV-2A catalytic sequence at the C-terminus of the small (S) CP, followed by insertion of GFP.[61] Further work involved the expression of versions of GFP that were designed either to be retained in the endoplasmic reticulum (ER) or to be secreted into the apoplastic space. The expected subcellular localizations were confirmed by confocal microscopy (Nicholson *et al.*, unpubl. data, 2005). This encouraged the use of the CPMV system for expression of valuable proteins such as small immune proteins (SIP) specific to the coronavirus transmissible gastroenteritis virus (TGEV), whose immunological properties are now being tested.

Table 2 Immunogenic proteins reported using the viral polypeptide expression system in plants

Polypeptide expression system	Expressed sequence	Plant used for infection	Species protected	Immunological properties	Reference
TMV	VP1 from the foot and mouth disease virus (FMDV)	*Nicotiana benthamiana*	Mouse	Parenteral application in the presence of CFA protected all animals in two separate experiments	47
TMV	38C13 scFv specific to the 38C13 mouse B-cell lymphoma	*Nicotiana benthamiana*	Mouse	Mice vaccinated with the affinity purified 38C13 scFv generated >10 µg/mL anti-idiotype immunoglobulins; these mice were protected from challenge by a lethal dose of the syngeneic 38C13 tumour	43
TMV	Betv1, a major birch pollen antigen	*Nicotiana benthamiana*	Mouse	Parenteral application with crude leaf extracts generated immunological responses comparable to those induced by the protein expressed in *Escherichia coli* or extracted from birch pollen	48
TMV	gDc from bovine herpes virus type 1 (BHV-1)	*Nicotiana benthamiana*	Mouse Cattle	Parenteral application of oil-based vaccines with crude extracts protected both animals	49
PVX	E7 from human papillomavirus 16 (HPV-16)	*Nicotiana benthamiana*	Mouse	Parenteral application with foliar extracts in the presence of Quil A as an adjuvant developed both antibody and cell-mediated immune responses	54
PPV	VP60 from rabbit haemorrhagic disease virus (RHDV)	*Nicotiana clevelandii*	Rabbit	Parenteral application with crude extracts in the presence of adjuvant fully protected rabbits against subsequent challenge with a lethal dose of RHDV	57
CPMV	Small immunogenic proteins ($\in$SIP) specific to transmissible gastroenteritis virus (TGEV)	*Vigna unguiculata*	Pig	Oral application using $\in$SIP infected crude leaf powder	Lomonossoff (unpubl. data, 2005)

CPMV, cowpea mosaic virus; PPV, plum pox virus; PVX, potato virus X; TMV, tobacco mosaic virus.

Conclusions

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 (Tables 1,2), in some cases in the target animals.[16,17,57] In most of these cases, immunity was stimulated by parenteral immunization, but there are encouraging signs that mucosal immunization may be possible.[25,29,62] 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 (i) CPMV,[61] clover yellow vein virus (CIYVV)[63] and pea early browning virus (PEBV),[64] all of which infect legumes; (ii) wheat streak mosaic virus (WSMV),[65] which infects cereals; and (iii) zucchini yellow mosaic virus (ZYMV),[66] which infects cucurbits – is likely to play a prominent role. In addition, the development of combined transgene/virus complementation systems, such as those described by Sanchez-Navarro *et al.*[67] and Mori *et al.*,[68] 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 is shown to be efficacious, there will still be a need to purify, at least partially, proteins and chimeric particles for certain applications. Although 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. To this end, results reported on the large-scale growth and purification of a TMV chimera expressing a 12 amino acid malarial peptide[69] show that although growth under field conditions gives a lower yield per gram (fresh weight) of tissue than growth in a greenhouse, an excess of 1 kg per acre of purified particles could potentially be obtained. Similarly, it has been 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.[70] The development of methods for the industrial-scale production of plant-derived vaccines will make an important contribution to the practical use of such material.

Acknowledgements

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