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The Potential of Plant Virus Vectors for Vaccine Production

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Abstract

Plants viruses are versatile vectors that allow the rapid and convenient production of recombinant proteins in plants. Compared with production systems based on transgenic plants, viral vectors are easier to manipulate and recombinant proteins can be produced more quickly and in greater yields. Over the last few years, there has been much interest in the development of plant viruses as vectors for the production of vaccines, either as whole polypeptides or epitopes displayed on the surface of chimeric viral particles. Several viruses have been extensively developed for vaccine production, including tobacco mosaic virus, potato virus X and cowpea mosaic virus. Vaccine candidates have been produced against a range of human and animal diseases, and in many cases have shown immunogenic activity and protection in the face of disease challenge. In this review, we discuss the advantages of plant virus vectors, the development of different viruses as vector systems, and the immunological experiments that have demonstrated the principle of plant virus-derived vaccines.

1. Introduction

Plants are beginning to gain a foothold in the competitive landscape of recombinant protein production systems. This is because of the numerous advantages of plants over conventional fermentation-based technologies – the low cost of large-scale production, the unlimited scalability, the ability of plant cells to carry out higher eukaryote post-translational modifications, and the absence of human pathogens.[1,2] After a series of successful proof-of-principle studies and early stage clinical trials, plants are finally attracting serious commercial interest, and high-profile international programmes for the development of plant-derived pharmaceuticals are underway.[3,4] Recombinant antibodies and vaccine candidates are at the forefront of this research and development programme, boosted by the recent approval of the first plant-derived vaccine for poultry, to be marketed by Dow AgroSciences.[5]

A wide variety of plant-based expression systems is now available to those interested in vaccine production. This reflects the many different candidate host species, and different systems based on organ-specific expression or secretion, plant cell cultures, microbial plants and aquatic plants grown in bioreactors.[6,7] There is also a choice of several gene delivery and expression mechanisms. Typically, the strategy is to create transgenic plants, i.e. plants with stable genetic modifications either in the nuclear or chloroplast genomes. While the establishment of permanent transgenic lines has its advantages, drawbacks include the long development time (required for transformation, regeneration, analysis and several generations of breeding to achieve a suitable number of plants for production) and concerns about transgene escape in the environment. An alternative strategy is transient gene expression, usually achieved by vacuum infiltration of leaves with *Agrobacterium tumefaciens*,[8] but this approach has limited scalability and is not suitable for large-scale production. There has also been some recent interest in the development of expression systems based on cultured plant cells,[9] although it has proven difficult to achieve high yields, and plant cells rely on fermentation technology similar to that used for mammalian cell cultures.

In this review, we discuss another plant-based system, the use of recombinant plant viruses to produce heterologous proteins *in planta*. Viral genomes are much more convenient to manipulate than plant genomes, and the infection of plants with recombinant viruses is simpler than regenerating transgenic plants. Potentially, plants carrying recombinant viruses can be grown on the same scale as transgenic plants, but with a much shorter development time. Although the transgene is carried on a viral genome rather than in the plant genome, the expressed protein is processed in the same manner as endogenous plant proteins, meaning that appropriate folding, targeting and post-translational modification of the protein is possible. The viral system is therefore uniquely simple, flexible and efficient, and has the potential for protein manufacture in both contained and open facilities.[10-12]

2. Overview of Viral Expression Systems

There are two major strategies for producing vaccine candidates using plant viruses: (i) the target antigen is engineered as a discrete reading frame in the viral genome and expression is (usually) directed by a subgenomic RNA promoter;[13] and (ii) target antigens are expressed as in-frame fusions with the viral coat protein, allowing the display of heterologous epitopes on the surface of the chimeric viral particle.[14] In the first strategy, the soluble heterologous protein is the product, and the virus particles and any endogenous plant proteins are discarded when the product is recovered. This is directly analogous to the situation that would occur in conventional transgenic plants (figure 1). In the epitope display system, the entire viral particle is the product, the chimeric virus particles are extracted from the plant, and the virus serves not only as an expression vector but also as a delivery vehicle to present the epitope to the immune system (figure 2). Such chimeric particles are easily purified and can enhance pathogen-specific immune responses because they present multiple copies of the epitope on their surfaces.[15]

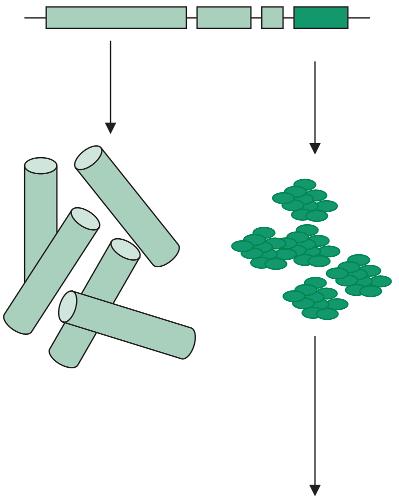

Fig. 1. A polypeptide expression strategy using plant viral vectors. In this approach, the heterologous polypeptide (in this context a subunit vaccine) is expressed as an independent transgene under the control of a separate promoter (usually the subgenomic coat protein promoter to maximise expression). The product, which is predominantly in the soluble fraction, is extracted from the plant material and purified. Virus particles (shown as cylinders) and other proteins are eliminated at this stage.

3. Strategies for Engineering Plant Viruses

Most plant viruses have positive-sense RNA genomes, and it is these viruses that have been the most widely exploited as expression vectors. DNA viruses were favoured in early experiments because cDNA copies of RNA genomes could not be generated and the fidelity of viral RNA-directed RNA polymerase was a concern. However, foreign sequences inserted into DNA viruses were often shown to destabilise the vectors. The use of DNA viruses as expression vectors has been largely abandoned now that cDNA copies of RNA viruses can be produced routinely. DNA viruses also have a restricted capacity for foreign DNA because they pack their genetic material into a pre-formed capsid. The only way to overcome this is to remove non-essential genes, which generally has a negative effect on virus accumulation. In contrast, RNA viruses build their capsid around the genome, and consequently there is less constraint on transgene size. The typical strategy is to add transgenes to the complete genome, preserving all the normal viral functions. The recombinant DNA copy of the genome is then either transcribed *in vitro* to yield infectious RNA that is used to inoculate plants, or introduced into the plant as an expression unit under the control of a plant promoter to yield viral genomic RNA *in vivo*. Other reasons for focusing on RNA viruses include their wide host range, high titres and the fact that RNA genomes introduced into plant cells are directly translated, yielding protein almost immediately.

A number of different plant viruses have been developed as expression and/or display vectors. The most widely used are tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), plum pox virus (PPV) and alfalfa mosaic virus (AlMV). Tomato bushy stunt virus (TBSV) and cucumber mosaic virus (CMV) have also been described as vectors for the expression of vaccine candidates in plants. Published studies involving the use of plant viruses to produce vaccine candidates are listed in table I.

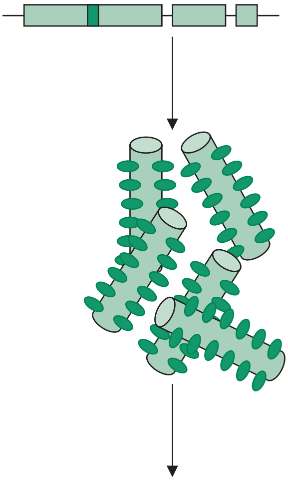

Fig. 2. The epitope display strategy using plant viral vectors. In this approach, the heterologous peptide is a vaccine epitope. The DNA sequence encoding this peptide is inserted at a suitable position within the coat protein gene as an in-frame fusion so that the peptide is displayed on the virus surface. If a purified product is required, the chimeric virus particles can be extracted from the infected plant material using standard protocols for plant virus recovery.

Table I. Vaccine candidates produced in plants using viral vectors

Production system	Antigen	Comments	References
TMV whole polypeptide expression in tobacco (*Nicotiana benthamiana*)	Foot-and-mouth disease virus VP1	Mice protected by parenteral administration in the presence of CFA	16
	38C13 scFv specific to the 38C13 mouse B-cell lymphoma	Mice vaccinated with purified 38C13 scFv generated anti-idiotype immunoglobulins and were protected from challenge by a lethal dose of the syngeneic 38C13 tumour. Phase I human clinical trials successful	17,18
	Betv1 (pollen antigen)	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	19
	gDc from bovine herpes virus type 1	Parenteral application of oil-based vaccines with crude extracts protected mice and cattle	20
	Hepatitis C virus epitope/cholera toxin B fusion	Antibodies elicited against both epitopes following nasal administration	21
TMV epitope display in tobacco (*N. tabacum*)	Murine hepatitis virus glycoprotein S 5B19 epitope	Immunogenic in mice following parenteral or nasal administration. Protected mice against virulent strain of the virus	22
	Murine sperm ZP3 protein epitope	Parenteral administration elicited specific antibodies	23
	Foot-and-mouth disease virus epitope	Parenteral administration provided protection in guinea pigs against pathogen challenge. Oral administration less effective	24
	Pseudomonas aeruginosa membrane protein F epitope	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with *P. aeruginosa*	25,26
	Porcine epidemic diarrhoea virus core neutralising epitope	No immunological data reported	27
TMV vector with AlMV coat protein epitope display in tobacco (*N. benthamiana*) and spinach	Rabies virus glycoprotein (G) and nucleoprotein (N)	Immunogenic and protective in mice when delivered orally and parenterally. Immunogenic in humans following oral administration	28-30
	HIV (type 1) gp120 protein	Neutralising antibodies produced. Immunogenic in mice following parenteral administration	28
TMV magnifection	*Yersinia pestis* F1 and V epitopes, and F1-V fusion	Systemic immune response following subcutaneous administration in guinea pigs. Protection against aerosol challenge with virulent *Y. pestis*	31
TMV biotinylated scaffold	Canine oral papillomavirus L2 protein	Viral particles more immunogenic in mice than uncoupled antigen	32
PVX whole polypeptide expression in tobacco (*N. benthamiana*)	Human papillomavirus (type 16) E7 protein	Immunogenic and protective in parenterally vaccinated mice	33
	Murine rotavirus VP6 protein	Immunogenic in mice following oral administration	34

Continued next page

Table I. Contd

Production system	Antigen	Comments	References
	Toxoplasma gondii SAG1	Mice vaccinated with SAG1 showed significantly lower brain cyst burdens compared with those from the control group following oral challenge with a nonlethal dose of the *T. gondii* Me49 strain	35
PVX epitope display in tobacco (*N. benthamiana*)	HIV (type 1) ELDKWA epitope	Neutralising antibodies produced. Immunogenic in mice following parenteral or nasal administration	36
	Staphylococcus aureus D2 epitope of fibronectin-binding protein	Specific antibodies produced. Immunogenic in mice and rats following parenteral, oral or nasal delivery	37
CPMV whole polypeptide expression in cowpea	Transmissible gastroenteritis virus peptides	No immunological data reported thus far	Cited in Brennan et al. [38]
CPMV epitope display in cowpea	Canine parvovirus VP2 epitope	Immunogenic in mice following parenteral or nasal administration. Protective against lethal challenge in parenterally immunised dogs	39,40
	Foot-and-mouth disease virus VP1 epitope	No immunological data reported	41
	Mink enteritis virus VP2 epitope	Immunogenic in mink following parenteral administration. Protective against viral challenge	42
	Human rhinovirus (type 14) VP1 epitope	Immunogenic in rabbits when delivered parenterally	43
	P. aeruginosa membrane protein F epitope	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with *P. aeruginosa*	26,44,45
	S. aureus D2 epitope of fibronectin-binding protein	Specific antibodies produced. Immunogenic in mice and rats following parenteral, oral or nasal delivery	37
	HIV gp41	Neutralising antibodies raised in mice	46,47
	Malarial peptide P109	Antibodies raised in rabbits	48
PPV whole polypeptide expression in tobacco (*N. clevelandii*)	Rabbit haemorrhagic disease virus VP60 epitope	Neutralising antibodies produced. Immunogenic in mice and rabbits following parenteral administration. Rabbits survived lethal challenge following parenteral administration	49,50
PPV epitope display in tobacco (*N. clevelandii*)	Canine parvovirus VP2 epitope	Neutralising antibodies produced in mice. Immunogenic in mice and rabbits following parenteral administration	50
AlMV epitope display in tobacco (*N. tabacum*) transgenic for AlMV RNA1 and RNA2	Respiratory syncytial virus g and F proteins	Neutralising antibodies produced. Immunogenic in mice following parenteral administration. Mice protected from viral challenge	51,52
TBSV epitope display in tobacco (*N. benthamiana*)	HIV (type 1) p120 protein	Neutralising antibodies produced. Immunogenic in mice following parenteral administration	53
	HIV (type 1) p24 protein	No immunological data reported	54
CMV epitope display in tobacco (*N. benthamiana*)	Hepatitis C virus HVR1 epitope of E2 envelope protein	Cross-reactive with a wide range of human anti-HVR1 antibodies	55,56

AlMV = alfalfa mosaic virus; **CFA** = complete Freund's adjuvant; **CMV** = cucumber mosaic virus; **CPMV** = cowpea mosaic virus; **HVR1** = hypervariable region 1; **PPV** = plum pox virus; **PVX** = potato virus X; **SAG1** = surface antigen 1; **scFv** = single-chain variable fragment (recombinant antibody); **TBSV** = tomato bushy stunt virus; **TMV** = tobacco mosaic virus.

4. Viruses Used as Expression Vectors

4.1 Tobacco Mosaic Virus (TMV)

TMV, the type species of the genus *Tobamovirus*, is the most extensively studied plant RNA virus and was one of the earliest to be developed as a vector. It has a monopartite genome approximately 6.5kb in length encoding at least four polypeptides, including a movement protein and a coat protein that are translated from subgenomic RNAs (figure 3). Several groups have demonstrated the feasibility of using TMV as an expression vector in plants and the first therapeutic recombinant protein expressed in plants using a viral vector, trichosanthin, was produced using a TMV-derived expression vector.[57]

The most widely used TMV vectors have an additional heterologous subgenomic promoter placed upstream of a cloning site for transgene insertion. The promoter is derived from a closely related tobamovirus to reduce the frequency of homologous recombination with the native coat protein promoter, which would result in elimination of the transgene.[58,59] Further modifications have been carried out to enhance cell-to-cell movement and stability, resulting in recombinant protein expression levels in some cases exceeding 2% of total soluble leaf protein.[60]

TMV has been particularly useful for the expression of recombinant antibodies, including single-chain variable fragments and whole immunoglobulins, the latter comprising two different polypeptides expressed from different vectors in a mixed infection.[61] Several immunogenic molecules have been expressed using TMV vectors including two proteins from animal pathogens,[16,62] a birch pollen antigen[19] and several anti-idiotypic single-chain variable fragment recombinant antibodies that are protective against B-cell lymphoma.[17,18] The latter were developed as patient-specific therapies by the US-based Large Scale Biology Company, and at least 12 molecules were submitted for phase I clinical trials before the company went into liquidation.[3,4]

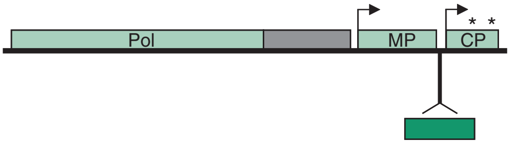

Fig. 3. Generic vector map of tobacco mosaic virus. Open reading frames found in the wild-type virus are shown as shaded boxes, encoding polymerase (Pol), movement protein (MP) and coat protein (CP). Arrows show the positions of subgenomic promoters. The grey box is an extension of the polymerase open reading frame accessed via a leaky stop codon. Asterisks show favoured peptide insertion sites for epitope display. The dark box represents a heterologous transgene, usually inserted between the MP and CP open reading frames and controlled by an additional subgenomic promoter.

TMV has also been developed as an epitope-presentation system because the wild-type virus particle contains 2130 copies of the coat protein, making it an attractive platform for peptide display. Detailed analysis of the TMV coat protein by x-ray crystallography identified several sites apparently suitable for the insertion of foreign peptides. Initial experiments were unsuccessful because the recombinant coat protein subunits did not assemble into stable virus particles. This problem was addressed by developing a system in which both wild-type and epitope-containing coat proteins combined to form heteromeric virus particles, achieved through the introduction of a leaky termination codon.[63,64] Sites have since been identified where small peptides (~20 amino acids) can be inserted into the coat protein without disrupting assembly, allowing the formation of recombinant particles in which every copy of the coat protein contains the epitope.[65] A variety of epitopes have been expressed using TMV vectors, including peptides from foot-and-mouth disease virus (FMDV)[24] and the opportunistic pathogen *Pseudomonas aeruginosa*.[25]

The restriction to ~20 amino acids allows only small peptide epitopes to be displayed on homomeric TMV particles. To circumvent this issue, Yusibov et al.[28] generated a new type of TMV vector in which the coat protein gene from alfalfa mosaic virus (AlMV) was expressed under the control of the TMV coat protein subgenomic promoter. This heterologous coat protein gene was modified to include the desired epitope, and thus far peptides of up to 47 amino acids in length have been incorporated successfully, which is larger than can be achieved with either TMV or AlMV alone. Two types of particles are produced, one composed of AlMV coat protein, which is used to display the epitope, and one composed of a tobamovirus coat protein, which facilitates long-distance movement of the hybrid virus. This system has been used to express epitopes from rabies virus and HIV on AlMV particles, both of which stimulated the production of neutralising antibodies in animal studies.[28] The rabies vaccine was also protective against lethal challenge with the virus in mice.[29]

Recently, a novel approach has been used to display larger polypeptides on the surface of TMV particles. Smith et al.[32] introduced a reactive lysine residue into the externally displayed N-terminus of the coat protein, allowing each subunit to be biotinylated. They then bound a model antigen (green fluorescent protein [GFP] conjugated to streptavidin) to the modified capsids, resulting in the display of up to 2200 GFP molecules per virion. The same principle was then applied to the display of an N-terminal fragment of the canine oral papillomavirus L2 protein. The use of TMV as a scaffold for polypeptide display significantly enhanced the immunogenicity of the L2 protein compared with the free antigen when administered to mice.

4.2 Potato Virus X (PVX)

PVX, the type species of the genus *Potexvirus*, has been used both as an expression vector and epitope presentation system, but is becoming more widely renowned as a tool for virus-induced gene silencing.[66] Like TMV, PVX has a monopartite RNA genome and it is organised in a similar fashion, with the coat protein expressed from a subgenomic promoter (figure 4). Consequently, its development as a vector has also followed the pattern established with TMV, i.e. the addition of a second subgenomic promoter used to express the protein of interest. This system has been used to express various heterologous proteins including at least three vaccine candidates: the VP6 protein from a murine rotavirus,[34] the E7 oncoprotein from human papillomavirus[33] and the surface antigen of the toxoplasmosis parasite *Toxoplasma gondii*.[35] In this last report, the authors also described another vector in which the transgene replaced the virus movement protein gene, and another in which the transgene was expressed under the control of the cauliflower mosaic virus 35S promoter together with a signal peptide to target the protein to the apoplastic space.

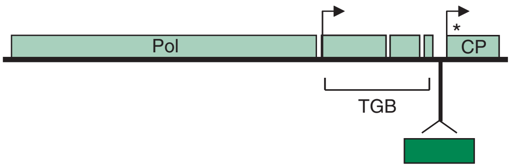

Fig. 4. Generic vector map of potato virus X. Open reading frames found in the wild-type virus are shown as shaded boxes: polymerase (Pol), triple gene block (TGB) and coat protein (CP). Arrows show the positions of subgenomic promoters. The asterisk shows the favoured peptide insertion site for epitope display. The dark box represents a heterologous transgene, usually inserted between the TGB and CP open reading frame and controlled by an additional subgenomic promoter.

Santa Cruz et al.[67] created an alternative PVX-based expression vector in which the transgene (in this case the gene for green fluorescent protein) was fused to the 5’ end of the coat protein gene via a FMDV 2A peptide sequence. This allows occasional ribosomal skipping,[68] such that the heterologous protein can be expressed both as an independent polypeptide containing a FMDV 2A peptide extension and as a fusion with the PVX coat protein. In addition to the subgenomic promoter strategy, O’Brien et al.[34] also used this system to express their murine rotavirus protein. They found that the independent VP6-2A proteins assembled successfully into rotavirus-like particles, while the fusion proteins assembled to form PVX particles displaying the rotavirus protein on their surfaces. Although this is an elegant way to produce subunit vaccines, the C-terminal FMDV 2A peptide extension might conflict with regulatory requirements governing therapeutic proteins, which favour the expression of native polypeptides. To address this issue, Toth et al.[69] created a variant PVX vector in which an internal ribosome entry site is placed between the transgene and coat protein gene. This vector produces a bi-cistronic mRNA, from which both the coat protein and foreign protein are produced at detectable levels.

The ability of PVX particles to assemble and display large protein extensions shows that the virus could also be useful as an epitope display system. Marusic et al.[36] have used PVX to display an epitope of HIV p41, which stimulated mice to produce IgG and IgA antibodies against the virus. More recently, Uhde et al.[70] have shown that the PVX coat protein can accommodate more than one peptide, by expressing tandem epitopes from beet necrotic yellow vein virus (BNYVV). The pI value of recombinant coat proteins strongly influences the efficiency of particle assembly and mutations that introduce charge compensation can occur over serial passages if the isoelectric point is much higher than the wild-type coat protein value (U. Commandeur, unpublished data). This phenomenon has also been observed in recombinant TMV and CPMV epitope display systems.[65,71]

4.3 Cowpea Mosaic Virus (CPMV)

CPMV, the type species of the genus *Comovirus*, was the first plant virus to be developed as an epitope display system.[41] The virus has a well characterised structure in which 60 copies each of two coat protein subunits (L and S) are arranged to form an icosahedral capsid surrounding a bipartite RNA genome. The properties that make CPMV particularly suitable for epitope display include the availability of several peptide loops near the surface of both the L and S coat protein subunits, which allow the insertion of foreign epitopes (figure 5). The most widely used insertion site is the βB-βC loop on the small subunit protein, which is the most exposed.[43] The extensive development of CPMV as an epitope display system has been covered exhaustively in several more specialised reviews, most recently by Canizares et al.,[38] and the reader is referred to these articles for more details. The importance of CPMV in vaccine development is that many of the epitopes displayed on this virus appear to elicit strong immune responses. Many of the chimeric CPMV particles have been used in immunological assays and in many cases the particles have elicited neutralising antibodies and protection against disease challenge (reviewed by Lomonossoff and Hamilton[72]). Protective immunity has been achieved against a range of pathogens including canine parvovirus, mink enteritis virus and *P. aeruginosa*.[39,42,44,45]

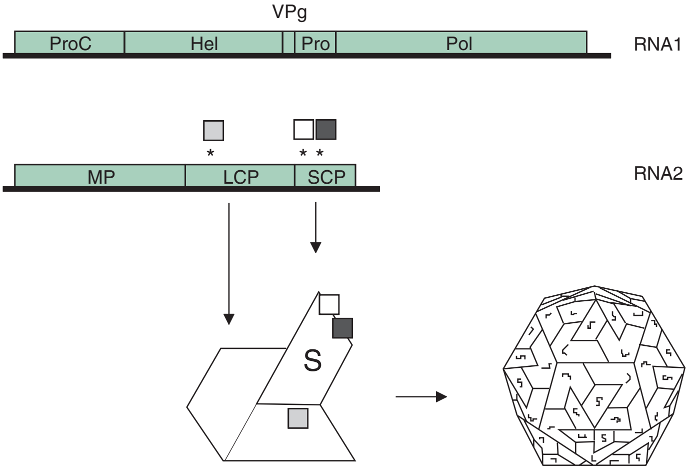

Fig. 5. Generic vector map of cowpea mosaic virus. Open reading frames found in the wild-type virus are shown as shaded boxes: ProC (proteinase cofactor), Hel (helicase), VPg (virus protein genome-linked), Pro (proteinase), Pol (polymerase), MP (movement protein), LCP (large coat protein subunit), and SCP (small coat protein subunit). Asterisks show the three most widely used sites for peptide insertion into the coat protein subunits. The lower diagram shows how these epitopes are presented in the context of the assembled subunits on the virus particle. Grey square = βE-αB loop; white square = the most commonly used site, the βB-βC loop; and black square = βC'-βC'' loop.

CPMV has also been used to express full-length proteins. Heterologous proteins are expressed either as coat protein or movement protein fusions with an integral proteolytic cleavage site to allow the target protein to be released,[73] or as C-terminal fusions with the S protein, incorporating the FMDV 2A peptide.[74]

4.4 Plum Pox Virus (PPV)

Like the viruses discussed above, PPV (a member of the genus *Potyvirus*) has been developed both as an expression vector for whole proteins and as an epitope display system.[75] However, an important difference between PPV and the other viruses is that the monopartite genome encodes a single polyprotein from which all the individual virus proteins are released by proteolytic cleavage. The general strategy to express whole proteins using PPV has therefore been to bracket the transgene with recognition sites for the viral protease VPg. There has been only one report of a vaccine candidate expressed in PPV thus far, and that is the VP60 protein from rabbit haemorrhagic disease virus, which was inserted between the polymerase and coat protein coding regions.[49] Rabbits immunised with leaf extract containing VP60 demonstrated pathogen-specific immune responses that protected the animals against a lethal challenge with rabbit haemorrhagic disease virus. No rabbit haemorrhagic disease virus was detected in the livers of the surviving animals 2 weeks after challenge. The serological responses of animals vaccinated with plant extracts containing VP60 were almost as high as those of animals immunised with a commercial vaccine.

PPV is advantageous as an epitope display system because both the N-terminus and the C-terminus of the coat protein are thought to be exposed on the surface of the virus. Fernandez-Fernandez et al.[50] used the N-terminus to display single-copy and tandemly-repeated epitopes of the 6L15 antigenic peptide of canine parvovirus VP2 protein. The recombinant virus was as infectious as the wild type in tobacco plants and was able to induce high levels of neutralising antibodies in both mice and rabbits. The same group used the PEPSCAN algorithm to identify immunogenic hotspots in the virus coat protein, and showed that the site between amino acids 43 and 52 was the most effective in terms of inducing specific antibody responses against foreign peptides.[76]

4.5 Alfalfa Mosaic Virus (AlMV)

AlMV is the type species of the genus *Alfamovirus*, a tripartite RNA virus with a broad host range that is particularly useful for producing target antigens as coat protein fusions. As with PPV, the N-terminus of the coat protein is located at the surface of the virus and is suitable for the insertion of peptides without interfering with virion assembly. As discussed above, the AlMV coat protein gene has been used in TMV vectors for the presentation of foreign epitopes,[28] but more recently the same strategy has been used with vectors derived directly from AlMV itself. This has been facilitated by the availability of P12 transgenic tobacco plants that produce the AlMV replicase from integrated and non-replicating DNA copies of RNA1 and RNA2, allowing the use of vectors based on RNA3 alone.[77] The virus particles produced in this system contain RNA3 only and are therefore non-infectious to other plants, providing an efficient containment strategy. In this way, Belanger et al.[51] used AlMV to display two peptides containing amino acids 174–187 of respiratory syncytial virus G protein. The particles generated strong B- and T-cell responses in primates.[52]

4.6 Tomato Bushy Stunt Virus (TBSV)

TBSV, type species of the genus *Tombusvirus*, has been explored as a potential vector for full-length polypeptides and epitope display. A basic vector, in which most of the non-essential coat protein gene was replaced by a cloning site, was constructed by Scholthof,[78] allowing Zhang et al.[54] to use the vector to express the HIV p24 antigen as an N-terminal fusion with the truncated coat protein. C-terminal fusions with the intact coat protein have been generated to facilitate TBSV epitope display. This strategy has been used by Joelson et al.[53] to display an epitope of HIV p120.

4.7 Cucumber Mosaic Virus (CMV)

CMV is the type species of the genus *Cucumovirus* and is closely related to AlMV, sharing its tripartite RNA genome and wide host range. Natilla et al.[55] developed a pseudorecombinant strain of the virus, comprising RNA3 from the CMV-S strain (containing the coat protein gene) and the RNA1 and RNA2 components from the CMV-D strain. The R9 mimotope of hepatitis C virus, a synthetic surrogate derived from a consensus profile of many hypervariable region 1 (HVR1) sequences of the putative hepatitis C virus envelope protein E2, was introduced into three separate sites in the coat protein gene. Serum samples from 60 patients with chronic hepatitis C displayed significant immunoreactivity to crude extracts from plants infected with these chimeric CMV particles.[55] Evidence was also obtained that the chimeric R9-CMV elicits a specific humoral response in rabbits.[56]

4.8 Zucchini Yellow Mosaic Virus (ZYMV)

ZYMV is a member of the *Potyvirus* genus and is related to PPV. Although this virus has not been used to express or display immunogenic peptides, it is worth noting that it has been used to express two plant proteins with activity against HIV, namely MAP30 and GAP31.[79]

5. Efficacy of Vaccine Candidates Produced Using Plant Viruses

Many ‘complete polypeptide’ vaccine candidates produced using plant viruses have shown immunogenic and protective efficacy in test animals. In some of these reports, the subunit vaccines comprised pathogen sequences alone, whereas in others the pathogen sequence was fused to molecules providing adjuvant or other stimulatory activity, such as the pentameric cholera toxin B subunit or even plant viral sequences.[80] Vaccine candidates have been evaluated either as purified recombinant proteins or in crude plant extracts. For example, Wigdorovitz et al.[16] demonstrated the efficacy of FMDV VP1 protein produced in tobacco using a TMV vector. The protein was administered intraperitoneally to mice as a leaf extract. All the immunised mice produced neutralising antibodies in response to the extract and were protected when challenged with virulent FMDV. One-year-old calves immunised with plant extracts containing VP1 also developed FMDV-specific antibody responses. Other immunological studies involving plant virus-derived complete polypeptide antibodies are listed in table I.

Although studies involving complete polypeptide vaccines have been successful, there have been relatively few in total. In contrast, a much larger number of immunological studies have been carried out using plant viruses displaying heterologous epitopes and these have demonstrated success in both veterinary and human clinical trials. For example, CPMV particles displaying a 17-mer neutralising epitope from the VP2 capsid protein of mink enteritis virus protected all the test animals against challenge with the virulent virus.[42] A modified construct that presented the peptide on the surface of both the L and S coat protein subunits induced an antibody response that was stronger than that of a peptide-keyhole limpet haemocyanin (KLH) conjugate.[40] The predominance of IgG-2a indicated early activation of T-helper type 1 cells (TH1). These results were validated by cell proliferation and interferon-γ release from murine cells exposed to virus particles *in vitro*. Intranasal immunisation resulted in a better mucosal response than serum response. These studies showed that it is possible to shift the bias towards a TH1 response (activation of macrophages and cytotoxic T cells) when peptides are presented on viral particles, and also that the recombinant viruses can protect against both systemic and mucosal infections.

CPMV particles have also been used to present epitopes from bacterial pathogens such as *P. aeruginosa* and *Staphylococcus aureus*. CPMV particles displaying the D2 peptide from the *S. aureus* fibronectin-binding protein induced high titres of fibronectin-binding protein-specific antibodies in mice and rats immunised subcutaneously.[37] Serum from the immunised mice inhibited fibronectin binding to immobilised recombinant fibronectin-binding protein, and rat serum was able to block the adherence of *S. aureus* to fibronectin. These studies show that vaccines from recombinant plant viruses could protect against *S. aureus* infections that include invasive endocarditis, septicaemia, peritonitis and bovine mastitis. A linear B-cell epitope from the outer membrane protein F of *P. aeruginosa* presented on CPMV particles induced peptide-specific antibodies in C57BL/6 mice that bound complement and increased phagocytosis of *P. aeruginosa* by human neutrophils *in vitro*.[44,45] In a mouse model of chronic pulmonary infection, the particles afforded protection when mice were challenged with two different subtypes of the pathogen. The levels of protection were similar to those observed when the peptide was coupled to KLH. In a study aiming to develop a potential cancer vaccine, CPMV particles were engineered to display a peptide derived from the epidermal growth factor receptor variant III. In this study,[81] it was shown that such particles elicited a peptide-specific antibody response in mice that protected the mice from tumour challenge. AlMV particles that contained an epitope from the G protein of the human respiratory syncytial virus elicited an immune response in mice and induced protective immunity against the virus.[51]

Most infectious diseases initially involve colonisation or invasion through mucosal surfaces by the pathogen. Therefore, as the first line of defence it is important to develop a strong mucosal immune response. Such responses can be achieved when the oral or nasal route is used for immunisation. Oral vaccines need to be formulated so they are protected from stomach acids and proteolytic enzymes in the gastrointestinal tract. The alternative method of intranasal immunisation has been shown to be effective at lower doses; the viral particles are more stable in this environment. Intranasal administration of recombinant CPMV particles elicited immune responses at distal sites, and antibodies could be detected in bronchial, intestinal and vaginal lavages.[40]

Intranasal immunisation of mice with chimeric TMV particles displaying the 5B19 epitope from the spike protein of murine hepatitis virus[22] elicited high IgG titres and moderate IgA titres specific to the peptide. Studies showed that longer periods of immunisation were more efficient than shorter periods at protecting the animals from challenge. Five of six mice immunised with chimeric TMV particles for 10 weeks survived intranasal challenge with murine hepatitis virus. However, only two of six mice that received immunisation for 6 weeks and one of six that received immunisation for 4 weeks survived the challenge.

Using a TMV vector, Nemchinov et al.[21] expressed the hepatitis C virus R9 mimotope discussed earlier, fused to the C-terminus of cholera toxin B. Intranasal delivery of plant extract that contained approximately 0.5–1μg cholera toxin B/HVR1 (i.e. <0.1μg of the HVR1 epitope), elicited anti-HVR1 antibodies in the absence of adjuvant. Intranasal immunisation of mice with chimeric PVX particles that presented H66, a neutralising epitope from HIV-1 p41, elicited high levels of HIV-1-specific IgG and IgA antibodies.[36] The anti-H66 IgG titres of these mice ranged from 2000 to >30 000, and anti-HIV IgA could also be detected in serum and faecal extracts.

Mice fed fresh spinach leaves that had been infected with AlMV particles displaying an epitope from the rabies virus showed an immune response against the peptide epitope. Serum IgG and IgA and mucosal IgA were detected.[29] Human volunteers (in US FDA-approved trials) fed with spinach containing recombinant particles generated peptide-specific IgG and IgA antibodies.[30] These trials also suggested that chimeric plant virus particles displaying peptide epitopes could be efficient in prime-boost vaccination regimens following immunisation with an alternative primary vaccine (e.g. a DNA vaccine).

6. New Approaches for the Development of Viral Vectors

There is continued effort to improve the quality of viral expression vectors, including the structure and yield of target molecules. Several investigators have attempted to increase the yields of recombinant protein by modifying the virus to improve its normal functions, e.g. improving the TMV movement protein through DNA shuffling.[82] The combination of components from different viruses can also lead to improved qualities, such as the development of a TMV vector modified to express AlMV coat protein, which can replicate in spinach and soybean even though the parent vector cannot.[83]

Other investigators have moved away from the intact virus strategy by transferring or replacing some of the intrinsic viral functions. One good reason for doing this is that it simplifies vector development, particularly for viruses with multipartite genomes. A useful approach is to delete essential viral genes and supply the missing products from a transgenic host plant, e.g. the P12 tobacco line discussed in section 4.5, which is transformed with non-replicating cDNA copies of AlMV RNA1 and RNA2. The host plant expresses AlMV replicase and therefore allows the replication of vectors based on AlMV RNA3 alone.[77] Similar two-component or complementation systems have been developed for TMV and other viruses.[84,85] An added advantage of these systems is that they provide biological containment. Intact viral vectors have the potential to spread and infect non-target plants, whereas replication-defective or movement-defective derivatives will only be able to propagate themselves in the appropriate complementary transgenic plant line.

Another reason for transferring viral functions away from the virus is that many of these functions are limiting when it comes to improving the efficiency of heterologous protein expression. These functions can be replaced with analogous functions of non-viral origin. The infection stage, for example, is generally very inefficient and is usually replaced by mechanical abrasion and inoculation or the direct delivery of viral nucleic acid into the plant via particle bombardment or by agroinfection (a term used to describe the delivery of viral genomic DNA, or cDNA if the virus has an RNA genome, to a plant using *Agrobacterium tumefaciens*). TMV vectors can be delivered to plants in this manner as cDNA copies under the control of a plant promoter. Nuclear expression of the cDNA yields RNA genomic transcripts, which then replicate and spread throughout the plant.[86] The deconstruction of plant virus vectors is taken a step further with the magnifection strategy, in which the systemic spreading functions of the virus are also rendered unnecessary through the use of *A. tumefaciens.*[87,88] In this system, the bacterium delivers the viral genome to so many cells that local spreading is sufficient for the entire plant to be infected. Like the infection stage, systemic spread is a limiting function, often one of the primary determinants of host range. Taking the systemic spreading function away from the virus and relying instead on the bacterium to deliver the viral genome to a large number of cells allows the same viral vector to be used in a wide range of plants. The system has been used to express plague antigens in *Nicotiana benthamiana,* which, when administered subcutaneously to guinea pigs, protected the animals against aerosol challenge with virulent *Yersinia pestis.*[31] The pros and cons of intact viruses and deconstructed viruses as vectors have been discussed in a recent review.[89]

7. The Future

The production of vaccines in plants offers several potential advantages over current commercial production systems for vaccines, including economy, scalability and increased safety, together with the possibility of oral delivery in edible plant tissues. Plant viruses provide even greater benefits because they are simple to manipulate, they shorten the product development timescale, and they offer potentially very high yields. Future development will need to focus on two areas – the optimisation of the current generation of vectors and the development of new vectors that can be used in appropriate hosts. Many of the viruses on which the current generation of vectors are based replicate most efficiently in specific host plants, predominantly tobacco (*Nicotiana tabacum*) and its close relative *N. benthamiana*. These vectors will be suitable for the production of vaccines that need to be purified prior to administration (and indeed most studies thus far have investigated the effects of parenteral immunisation), and studies should focus on improving yields and maintaining a high level of biosafety. A continued trend towards two-component systems and the use of defective viral replicons would be beneficial here. For oral delivery, particularly in developing countries, vectors should be developed that can infect suitable host plants, including cereals and legumes. Such vectors have been reported, e.g. an expression vector derived from wheat streak mosaic virus (WSMV), which infects cereals.[90] Alternatively, for plants susceptible to agroinfection, it may be simpler to use the magnifection system to overcome restrictions of host range caused by an inability of the virus to spread. Many plant viruses can replicate in cells from a large range of plant species and the ability of the *Agrobacterium*-based system to deliver the viral genome to a high percentage of cells could circumvent the limitations imposed by cell-to-cell movement and long-distance movement. It is also important in the future to focus on the development of an integrated production system, which brings together vector construction, propagation of plants and their infection, and, where necessary, downstream processing, extraction and purification of the target protein.

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