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Plant Viral Vectors as a Tool for Recombinant Vaccine Production

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ABSTRACT

Over the last few years the development of plant viral vectors for protein expression has made rapid and impressive progress. Plant viruses are versatile vectors for production of proteins since they are easy to manipulate, quick to evaluate and offer the possibility of great production yields. They are particularly powerful expression systems for the production of recombinant proteins and peptides for vaccination, since they are able to produce antigens alone or conjugated to viral capsid. Several viral vectors have been developed for vaccine production, mainly *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX). In this review, we will discuss the advantages of the use of plant viral vectors as expression vectors, the principal vectors that have been developed to date, and the different strategies used for expression, with emphasis on recent research conducted in Argentina.

Keywords: agroinfiltration, antigen molecular farming, RNA virus

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INTRODUCTION

The use of plants as biofactories for the production of vaccine antigens is becoming a very promising field. The majority of the recombinant proteins currently commercialized are produced in bacteria and mammalian cells, but conventional fermentation technology has limited scalability and high production costs that can be potentially overcome by plant biofactories, which have relative low production cost, are easy to scale-up and require little capital investment, since the infrastructure for plant cultivation and harvesting already exist (Twyman *et al.* 2005). In addition to this, the production of recombinant proteins in plants reduces the health risk of pathogen contamination since plant pathogens are not hazardous to human or animal health.

Three plant-based expression systems are now available for vaccine production: (1) stable transformation of the plant nuclear genome, (2) stable transformation of the chloroplast genome and (3) viral transient expression. In stable transformation strategies an expression cassette harbouring the gene of interest is integrated into the nuclear genome by non-homologous recombination or into the chloroplast genome by homologous recombination. In this way, the gene of interest is inherited over generations and the transgenic lines obtained can be easily propagated and scaled up for production. However, stable transformation technology is time-consuming and, since the integrated gene is genetically inherited, the possibility of a transgene escaping to the environment becomes a concern. In addition, nuclear transformation usually leads to low yields mainly due to post-transcriptional silencing of the gene of interest and positional effects. Chloroplast transformation, on the other hand, seems more robust in its capacity to produce high levels of recombinant proteins in a consistent manner. Nevertheless, the use of chloroplasts as transformation targets is limited to proteins that do not require post-transcriptional modifications for its biological activity, since these organelles, like bacteria, lack the eukaryotic post-transcriptional modification machinery (Bock 2007).

In transient viral expression the gene of interest is integrated into the viral genome and the modified virus is used to infect the host plant. Once inside a plant cell, the viral genetic material is not integrated into the host genome but undergoes several rounds of autonomous replication, amplifying the number of copies of the viral genome inside the host cell and potentially leading to high levels of expression of the gene of interest inserted into the vector. In addition, plant viruses are relatively small and easy to manipulate, and infecting plants with a recombinant virus is far easier than generating a transgenic plant. All these features make the use of transient viral expression a simple, flexible and efficient system to produce recombinant proteins in plants (Pogue *et al.* 2002).

In this review we will first characterize the types of

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plant viral vectors and then describe recent advances on the use of two of the most important of these vectors: the *Tobacco mosaic virus* and *Potato virus X*. The uses of gene silencing suppressors and plants that are transgenic for viral vectors will also be discussed.

PLANT VIRAL VECTORS

Plant viruses can have either DNA or RNA genomes. The development of plant viruses as vector expression systems started in the early 1980s using viruses with DNA genomes. The first plant virus vector was based on the *Cauliflower mosaic virus* (CaMV), a double stranded DNA virus, but the limited packaging capacity and the restricted amount of viral DNA that can be removed without affecting its functionality hampered its use as an expression system (Fütterer *et al*. 1989).

Most plant viruses have positive-sense RNA genomes, but they were not used as vectors until methodological advances in cDNA technology became suitable to convert large sequences of RNA into DNA. The most promising results using viral expression systems have been obtained with vectors based on *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX) genomes, all of which are RNA viruses.

First-generation vectors are functional viruses that express all the viral genes in addition to the gene of interest and cause systemic infections in the host plant (**Fig. 1**). The delivery of these vectors to the plant cells is made by mechanical infection with mature virions or infectious nucleic acid copies of the virus or by infiltration of plant tissue with an *Agrobacterium tumefaciens* strain harbouring the virus vector ("agroinfiltration"). From the point of initial infection the viral particles carrying the gene of interest have to spread all over the host plant to cause a systemic infection and assure high levels of expression. As a consequence, in first generation vectors the insertion of the gene of interest into the vector should not interfere with the functions of the viral proteins, since the vector needs to spread inside the host.

In first generation vectors the recombinant protein can be expressed from a strong independent viral promoter, usually the coat protein (CP) promoter (gene insertion vector) or as an in-frame fusion to the viral CP (peptide display vectors). Gene insertion vectors produce a soluble heterologous protein which can be extracted and purified from the rest of the plant and viral proteins. In this case the purification of the heterologous protein is similar to the purification of a protein expressed by conventional transgenic plants. Peptide display vectors provide an expression system in which the virus and the host plant can generate large quantities of the fusion protein and chimeric virus particles. These chimeric viral particles are then easily extracted and purified from the infected plants and can be used to enhance pathogen-specific immune responses as they present multiple copies of the epitope on their surfaces.

Gene insertion vectors have been successfully used to express several antigens, even though this kind of vector has showed some limitations that hamper the production of high yields of heterologous proteins. One of these limitations is the size of the gene that can be inserted without compromising the functionality of the virus vector. Another limitation is related to the promoter used for expressing the transgene. Since the gene of interest is usually inserted together with a copy of the strong promoter of the viral CP, the presence of duplicated promoter sequences can be a source of genetic instability for the vector, leading to the reversion to wild-type virus and the loss of the heterologous gene.

In contrast, the peptide display system does not have these limitations and can be very useful as far as the fusion proteins are compatible with the correct assembly of the virus. Work by different laboratories has shown that only small epitopes (e.g. fewer than 25 amino acids) can be expressed successfully as coat fusion proteins (Durrani *et al.* 1998; Cerovská *et al.* 2008), since longer peptides interfere with the correct assembly of viral particles. Some strategies have been established in order to overcome these assembly problems. One of them consists in producing a mix of fused and unfused CPs to allow a successful assembly of the virus-like particles. This can be achieved by two approaches. In the first one the 3′ terminus of the coat gene is modified so that the CP stop codon would function in a leaky way (Borovsky *et al.* 2006). In the other strategy the 2A peptide of the *Foot and mouth disease virus* (FMDV) is inserted between the epitope and the 5′ terminus of the CP gene in order to produce a ribosomal skip during translation (Santa Cruz *et al.* 1996; Donnelly *et al.* 2001). The other strategy utilizes a 15-aa-flexible linker fused to the C-terminal of the coat protein that allows the expression of a 133-aa fragment of protein A on the surface of a tobamovirus vector (Werner *et al.* 2006).

First generation vectors had several shortcomings, like the limited size of the genes that can be inserted into the vector and the necessity of not disturbing the functions of the viral genes in order to obtain systemic infection. Another problem with first-generation vectors is that they produce systemic infections with functional viral particles that can easily propagate to other plants, a serious concern from the point of view of biological containment. To circumvent some of these problems, second generation vectors were designed which, instead of using complete viral genomes, were built using only the viral sequences that are indispensable for an efficient infection. Two types of second-generation vectors exist: (1) gene replacement vectors and (2) modular or deconstructed vectors (**Fig. 1**).

Gene replacement vectors are generated by the substitution of a non-essential viral gene for the heterologous gene of interest. The lack of some genes causes the virus to be unable to spread through the host and prevent it from causing a systemic infection. The use of these vectors was made possible by the development of the magnifection technique, which allows the large-scale infection of the whole plant by vacuum infiltration of an *Agrobacterium tumefaciens* strain harbouring the viral vector (Marillonnet *et al.* 2005). In this process, the bacteria assume the functions of primary infection and systemic movement, circumventing the need of using vectors capable of causing systemic infection. Most of the vectors developed following this strategy replace the gene of the CP for the gene of interest. The most successful vector developed using this strategy is based on a TMV that expresses the heterologous gene from the CP promoter (Marillonnet *et al.* 2005).

In modular or deconstructed vectors the gene of interest is also inserted into a vector that lacks many viral sequences essential for cell-to-cell spreading and systemic infection. However, in contrast to simple gene replacement vectors, the omitted viral functions are delivered either by co-infiltration with an *Agrobacterium* strain carrying a vector containing the sequence for the missing viral functions or by using a transgenic host plant that provides the desired functions. The complementation of viral functions allows for systemic infection to occur, but the chances of creating fully functional viruses that can escape to the environment is strongly reduced. The strategy of using deconstructed viruses was possible thanks to the understanding that not all the viral components need to be present in the same nucleic acid molecule, but that they may be split up into different modular elements that can work together in the infectious development as happens with natural viruses that carry multipartite genomes. Viruses with a multipartite RNA genome have been used based on this approach such as *Alfalfa mosaic virus* (AIMV), from the genus *Alfamovirus*, which has a tripartite RNA genome. The RNA3 of AIMV has been engineered into an expression viral vector by insertion of a subgenomic promoter directing green fluorescent protein (GFP) expression. This cassette has been used to inoculate P12 transgenic tobacco plants, stably transformed with RNA1 and RNA2, producing the protein of interest (Sanchez-Navarro *et al.* 1988). A similar strategy

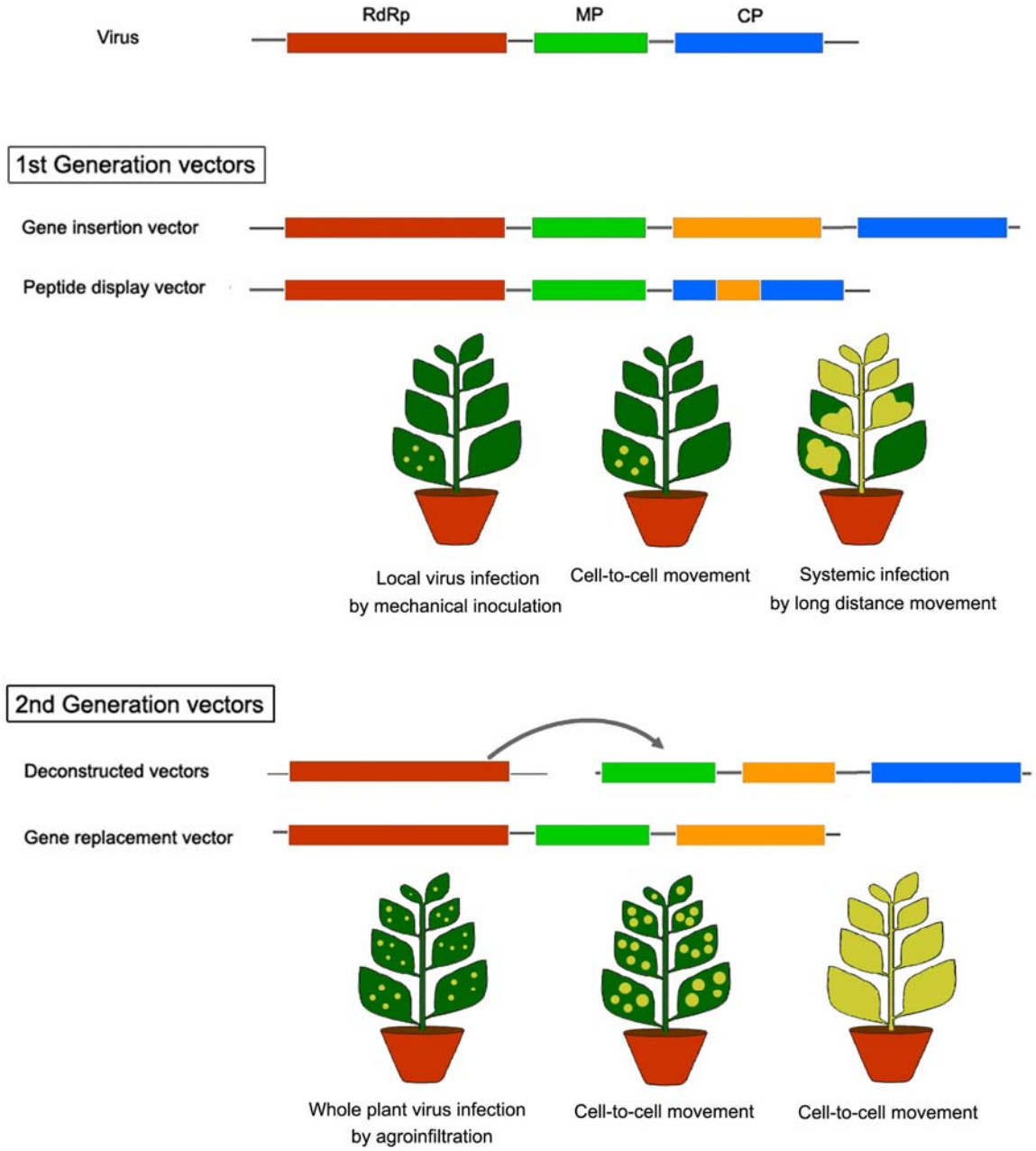

Fig. 1 Types of viral vectors and expression strategies. The scheme on the top shows a generic plant RNA virus with three essential genes: the viral replicase (RdRP), movement protein(s) (MP) and coat protein (CP). In the vector schemes below the heterologous gene is indicated by an orange bar. In first generation vectors, all viral genes are present and the heterologous gene can be inserted in the viral genome (gene insertion vector) or fused to the CP (display vectors). In second generation vectors, the viral genome is incomplete and can be complemented by a gene expressed from a plant transgene (deconstructed vectors). Alternatively, the heterologous gene can replace the CP. See text for details.

was used to produce two peptides of the *Human respiratory syncytial virus* as fusions with the AIMV CP (Belanger *et al.* 2000).

VIRAL VECTORS BASED ON *TOBACCO MOSAIC VIRUS* (TMV)

TMV, belonging to the genus *Tobamovirus*, is one of the most widely studied RNA virus and was one of the first to be successfully used as an expression vector in plants. Its monopartite genome encodes an RNA-dependent RNA polymerase, a movement protein and a CP. Gene replacement, gene insertion and peptide display strategies have all been developed based on TMV (**Fig. 2**).

The most widely used TMV-based vector is a gene insertion vector where the gene is expressed from a heterologous subgenomic CP so as to avoid the instability caused by homologous sequence recombination. Large Scale Biology Corporation (LSBC) developed a plant viral expression system based on this TMV gene insertion vector, which was used to produce many recombinant therapeutic proteins and vaccine antigens in plants. At least 12 molecules were submitted to phase I clinical trials including *Feline parvovirus* antigen, Non-Hodking's lymphoma antibody and a therapeutic *Human papilloma virus* vaccine before the company went to liquidation in December 2005 (Ma *et al.* 2005; McCormick *et al.* 2008; Spök *et al.* 2008). Several research groups have also utilized a TMV-insertion vector to produce different antigens like a *Dengue virus* envelope protein (Saejung *et al.* 2007), which was expressed in *Nicotiana tabacum* plants and shown to be able to produce neutralizing antibodies in mice against *Dengue type 2 virus*. Another antigen produced in a TMV-based vector was the colorectal cancer antigen GA733-2 (Verch *et al.* 2004). Importantly, this cancer antigen produced in tobacco plants elicited humoral and cellular responses that were similar to that elicited by the same antigen produced in insect cells, which is the one currently used in research and clinical

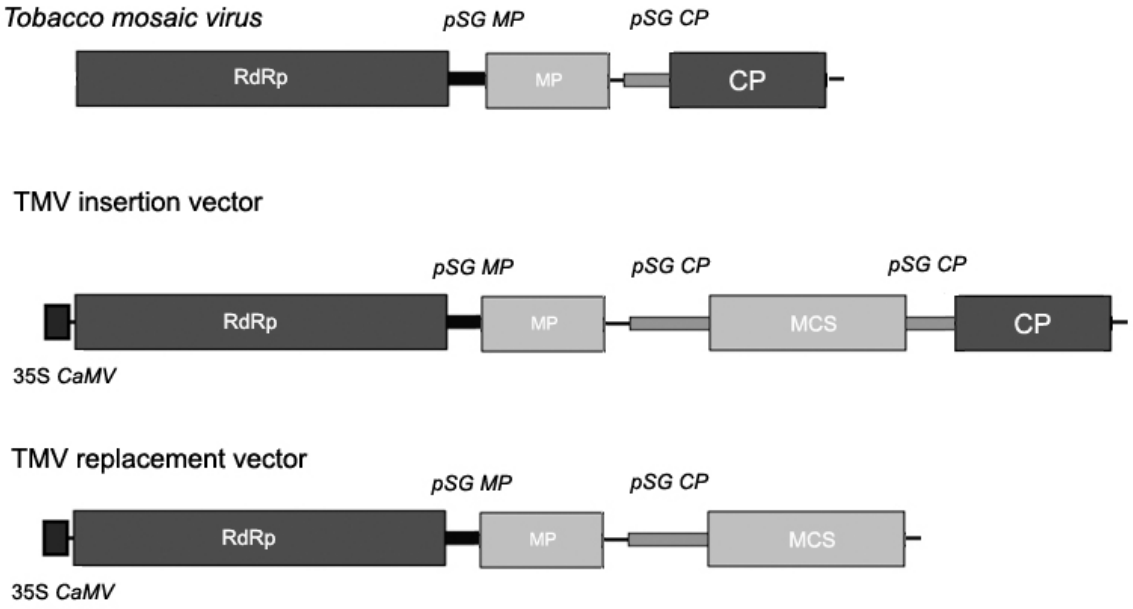

Fig. 2 ***Tobacco mosaic virus*** **(TMV) vectors.** Two different strategies of expression are represented, a generic insertion vector and a generic replacement vector (Large Scale Biology Corp.; Marillonnet *et al.* 2005). RdRP: viral replicase; MP: movement protein; CP coat protein; pSG: subgenomic promoter; 35S *CaMV* 35S promoter of the *Cauliflower mosaic virus*; MCS: multiple cloning site.

trials.

In Argentina, studies on the development of plant viral vectors as a tool for vaccine production were pioneered by Manuel Borca's team. In 1999, his group published the first report showing the expression of a FMDV antigen using a TMV-based vector (Wigdorovitz *et al.* 1999). The TMV genome was altered by inserting a polylinker sequence downstream of the coat subgenomic promoter in order to permit the insertion of the VP1 gene of FMDV. The expression of the viral CP gene itself was regulated by the insertion of a second homologous subgenomic promoter from a related virus. In this work, they demonstrated that mice immunized with foliar extracts from infected plants developed a protective immune response against experimental challenge with virulent FMDV. This TMV-based expression vector has been also used to express other heterologous proteins including the cytosolic form of the bovine herpesvirus type 1 (BHV-1) protein glycoprotein D (gDc) (Pérez Filgueira *et al.* 2003). In this case, it was observed that cattle vaccinated with plant-produced gDc showed good levels of protection after being challenged with the virulent BHV-1 and that virus excretion in these animals was comparable to cattle vaccinated with a commercial BHV-1 vaccine (Pérez Filgueira *et al.* 2003). The same group later developed an improved TMV-based vector (TMV-30B-HISc) with a stretch of seven histidine residues fused to the C-terminus of the foreign protein in order to facilitate its purification by immobilized metal affinity chromatography (Pérez Filgueira *et al.* 2004a). The TMV-30B-HISc vector was used to express VP8*, a trypsin-derived, 25-kDa fragment of the VP4 protein from bovine rotavirus (BRV) strain C-486. Recombinant VP8* protein could be expressed and readily purified from infected *N. benthamiana* plants and was used to immunize mice, which revealed that the antigenic characteristics of recombinant VP8*-His protein were intact. Moreover, immunization of mouse dams with recombinant VP8*-His conferred immune protection to their suckling pups upon a challenge with the BRV virus, showing that plant-produced antigen was capable of inducing passive protection in new-born mice through the immunization of their dams (Pérez Filgueira *et al.* 2004a). Production of the structural VP1 protein from FMDV and the core protein, p24, from *Human immunodeficiency virus 1* (HIV1) in *N. benthamiana* through the use of TMV-30B-HISc vector has also been reported (Pérez Filgueira *et al.* 2004b).

An impressive enhancement of expression levels achieved with a TMV-gene replacement vector was developed by mutating spurious splicing sites and introducing introns in the replicase sequence that increased the efficiency of initiation of viral replication (Marillonnet *et al.* 2005). By delivering this vector in *Nicotiana benthamiana* and *N. tabacum* by magnifection, it was possible to achieve yields of 4 g of GFP protein per kg of fresh leaf biomass in *N. benthamiana* and 2.5 g/kg in *N. tabacum*, representing 25-40% of total soluble protein. This vector has been used to develop a versatile expression system based on the *in planta* assembly of functional viral vectors from separate pro-vector modules (Marillonnet *et al.* 2004). They used agrobacteria to deliver several modules that are assembled inside the cell with the help of a site specific recombinase. In this way it is possible, for example, to test different subcellular localization signals or different fusion proteins by the use of pre-fabricated genetic modules, avoiding the time-consuming vector engineering process. This system has been used to express several therapeutic and antigens proteins like *Human growth hormone* and *Yersinia pestis* antigens with very good results (Gleba *et al.* 2007; Santi *et al.* 2006; Gils *et al.* 2005).

Musiychuk *et al.* (2007) have developed a powerful system based on a modified TMV replacement vector that allows the expression of several antigens fused to the *Clostridium thermocellum* thermostable enzyme lichenase (LicKM) from the subgenomic CP promoter. Small to full-length proteins can be fused to LicKM and expressed as N- or C-terminal fusions and/or internal fusions, a feature that permits the simultaneous expression of multiple targets. The thermostability of the LicKM is exploited for the easy and cost-effective recovery of the antigens because it is possible to remove up to 50% of contaminant plant proteins after a brief heat treatment. Using this approach more than 30 antigens from different pathogens have been successfully expressed, including antigens from *Trypanosoma brucei*, *Bacillus anthracis*, *Respiratory syncytial virus*, *Avian influenza A virus* and *Yersinia pestis* among others (Mett *et al.* 2007; Chichester *et al.* 2007; Shoji *et al.* 2008; Chichester *et al.* 2009; Shoji *et al.* 2009).

Peptide display techniques allow TMV to be used as a scaffold for the display of antigens for vaccine applications. One of the first examples of using TMV-peptide display vectors was the expression of malaria B-cell epitopes using a leaky stop signal to obtain a chimeric virion composed by a mixture of wild type CPs and peptide fused-CPs (Turpen

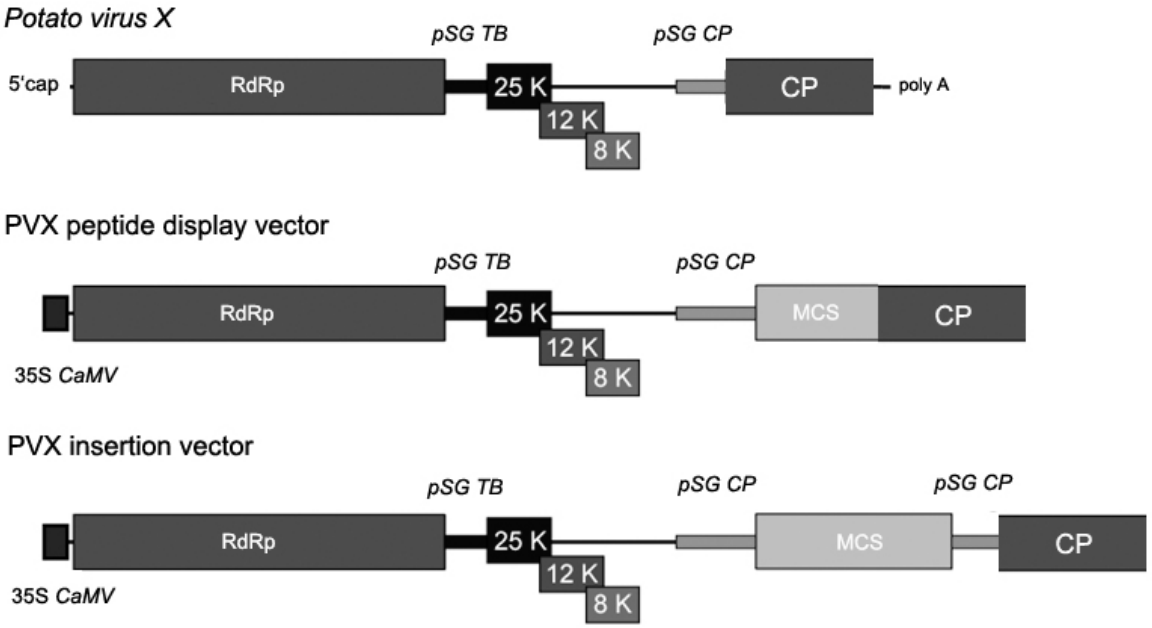

Fig. 3 ***Potato virus X*** **(PVX) vectors.** Two different strategies of expression are represented, a generic peptide display vector and a generic insertion vector (Chapman *et al.* 1992; Angell and Baulcombe 1997). RdRP: viral replicase; TB: Triple gene block encoding movement proteins 25K, 12K and 8K; CP: coat protein; pSG: subgenomic promoter; 35S *CaMV* 35S promoter of the *Cauliflower mosaic virus*; MCS: multiple cloning site.

et al. 1995). Using the same strategy two epitopes of the influenza virus hemagglutinin and one from HIV1 envelope protein were produced and successfully assembled (Sugiyama *et al.* 1995). Later, sites of the TMV CP were identified that allowed the assembly of virions containing 100% of chimeric CPs, which were used to produce epitopes of the FMDV and *Pseudomonas aeruginosa* (Gilleland *et al.* 2000; Staczek *et al.* 2000; Wu *et al.* 2003; Jiang *et al.* 2006). Unfortunately, the size of peptide that can be expressed by this approach is limited to around 20 amino acids, since longer peptides impair appropriate assembly of viral particles. This problem was overcome using an elegant strategy consisting in the modification of the TMV CP by introducing a reactive lysine at its N-terminal that is used to biotinylate the capsid. The heterologous protein is independently expressed in plants as a fusion with streptavidin and used to decorate TMV-biotinylated particles (Smith *et al.* 2006). Using GFP as a model protein the authors produced TMV particles harbouring approximately 2200 GFP molecules per virion, thus potentially expanding the utility of TMV as a scaffold for antigen display. The same approach was used to express a fragment of the *Canine oral papillomavirus* L2 protein. TMV displaying L2 protein fragment was significantly more immunogenic than the uncoupled antigen (Smith *et al.* 2006). Another work by the same authors demonstrated that TMV particles conjugated with different tumour antigens are effective carriers to stimulate *in vivo* cellular responses by γ-interferon and to improve protection against tumour challenge (McCormick *et al.* 2006a). An improvement of vaccine efficacy of TMV-conjugated particles was achieved by the conjugation in the same particle of an antigen peptide and a second peptide designed to serve as T helper epitopes or peptides that improve uptake (McCormick *et al.* 2006b).

VIRAL VECTORS BASED ON *POTATO VIRUS X* (PVX)

PVX belongs to the genus of *Potexvirus* and it has a monopartite RNA genome which encodes five polypeptides, including a RNA-dependent RNA polymerase, three movement proteins and a CP. The first PVX vectors were developed as insertion and replacement vectors using similar strategies that TMV vectors (Chapman *et al.* 1992) (**Fig. 3**). Since the CP of PVX is essential for cell-to-cell movement and systemic infection, earlier efforts to develop PVX as a replacement vector were unsuccessful (Chapman *et al.* 1992). PVX-based insertion vectors have been used to express some vaccine antigens and antibodies as *Human papilloma virus* E7 proteins (Franconi *et al.* 2002), *Hepatitis B virus* (HBV) nucleocapsid protein (Mechtcheriakova *et al.* 2006) and a derivate antibody against *Transmissible gastroenteritis virus* (TGEV) (Monger *et al.* 2006). This derivate antibody, formed by a single-chain antibody (scFv) against porcine TGEV linked to a domain from human immunoglobulin E (IgE), was able to passively immunize pigs against TGEV when supplied orally (Monger *et al.* 2006).

Our group in Argentina has developed a series of PVX vectors based on the infective clone obtained from a Latin American isolation (Orman *et al.* 1991). Using PVX insertion vectors we have expressed two antigens from *Toxoplasma gondii*, SAG1 and a truncated version of the dense granule protein 4 ($Gra4_{163\text{-}345}$) in *Nicotiana tabacum* leaves (Clemente *et al.* 2005; Ferraro *et al.* 2008). Plant produced-SAG1 and $Gra4_{163\text{-}345}$ were recognized by antibodies present in *Toxoplasma* seropositive human serum. In addition a protective response was achieved in mice immunized with tobacco leaf extracts expressing the SAG1 protein (Clemente *et al.* 2005).

Different strategies have been developed to use PVX as a peptide display vector. The first one consists in expressing the heterologous protein fused to the FMDV 2A peptide sequence and the N-terminal CP consecutively (Santa Cruz *et al.* 1996). The FMDV2A peptide produces a ribosomal 'skip' from one codon to the next without the formation of a peptide bond and allowing the translation of the sequence downstream (Donnelly *et al.* 2001). As result, unfused CP as well as CP fused to the heterologous protein are produced, allowing the assembly of chimeric PVX virions. Some antigens produced using this strategy are *Classical Swine Fever Virus* (CSFV) E2 glycoprotein (Marconi *et al.* 2006) and the VP6 antigen from a murine rotavirus (O'Brien *et al.* 2000). In our lab we have used this approach to express the ESAT-6 antigen from *Mycobacterim bovis* obtaining high-levels of expression (Zelada *et al.* 2006).

Small peptides have been expressed as simple N-terminal fusions to the CP of PVX with mixed results (**Fig. 3**). Lico *et al.* (2006) have systematically studied the effect of amino acid composition of the heterologous peptide and the isoelectric point of the N-terminal of the PVX CP in order

to define rational rules for the design of more effective peptide display vectors. A similar work was realized using epitopes of *Beet necrotic yellow vein* virus that confirm that the pI and charge value of the fused CP should be taken into consideration and adjusted by the addition of charge-compensating residues to obtain stable and properly assembled chimeric particles (Uhde-Holzem *et al.* 2007). A successful example of a peptide displayed on the surface of PVX is the high-yield expression of a neutralizing epitope from HIV-1 (Marusic *et al.* 2001). Chimeric particles administered via different routes in mice were able to elicit high levels of HIV-1-specific immunoglobulin G (IgG) and IgA antibodies with neutralizing capacity.

Another interesting approach takes advantage of the well-known characteristics of the *Cucumber mosaic virus* (CMV) CP as a peptide display system and combines it with the high levels of expression achieved with PVX vectors. The CMV CP was fused to the neutralizing epitope of *Newcastle disease virus* (NDV) and expressed in plants from a PVX-based vector and the resulting virus-like particles were shown to be an efficient carrier for vaccine epitopes (Natilla *et al.* 2006; Natilla and Nemchinov 2008). The authors also successfully expressed the extracellular domain of *Avian influenza A virus* M2 protein (M2e) in this system (Nemchinov and Natilla 2007). Applying a similar rationale, Cerovská *et al.* (2008) simultaneously expressed two epitopes of *Human papilloma virus* on the surface of *Potato virus A*. The CP of the *Potato virus A* was engineered to have an N-terminal fused to an epitope of the minor protein of L2 and a C-terminal fused to an epitope derived from E7 protein. The gene for the chimeric CP was cloned into a PVX-insertion vector and transiently expressed in plants using *Agrobacterium tumefaciens*-mediated inoculation.

USE OF POST-TRANSCRIPTIONAL SILENCING SUPPRESSORS

Irrespective of the type of vector and strategy used, the expression of foreign proteins in plants with viral vectors has to face one of the most important defense mechanisms in plants, post-transcriptional virus-induced gene silencing (VIGS). VIGS is a special protective mechanism of the plant elicited by viral infection that leads to the degradation of foreign RNA in the cell (Ruiz *et al.* 1998; Lu *et al.* 2003). When virus-induced gene silencing is triggered by the presence of a replicating viral vector there is a significant reduction of viral infection and productivity leading to low accumulation levels of the protein of interest.

To evade plant defence mechanisms, many viruses encode silencing suppressors that inhibit VIGS. Since these proteins act by a general suppression of silencing within plant cells in a non-specific manner, there was a possibility that these silencing suppressors could be used to enhance and extend the time of foreign gene expression in plants (Scholthof 2007). One of the first to use a silencing suppressor for increasing protein expression was Voinnet *et al.* (2003). They described a system based on the co-expression of an amplicon and a viral-encoded suppressor of gene silencing, the p19 protein from *Tomato bushy stunt virus* (TBSV) (Voinnet *et al.* 2003). This report showed that the simultaneous expression of p19 prevented the onset of post-transcriptional gene silencing (PTGS) in the infiltrated tissues and allowed for high expression levels of transient target proteins. Dorokhov *et al.* (2006) applied this approach to enhance the expression of a TMV insertion vector to express several *Mycobacterium* antigens.

A viral vector for efficient production of foreign proteins based on PVX was also developed by Atabekov's group (Komarova *et al.* 2006). This system used another silencing suppressor, HC-Pro from *Potato virus A* (PVA), to improve the efficiency of protein production. Recently, different approaches for transient expression using silencing suppressors have been developed (Azhakanandam *et al.* 2007; Lindbo 2007). In the near future the co-expression of suppressor proteins will be also a useful tool for expressing foreign proteins in transgenic plants for vaccine production.

STABLE EXPRESSION OF VIRAL VECTORS IN TRANSGENIC PLANTS

The use of transient expression of viral vectors by the magnifection technique as described in the previous sections is quick and powerful, in particular for the stage of research and development of the appropriate constructs. However, its industrial application has to deal with the problems of managing big volumes of bacteria needed for infecting plants and the limited amount of plants that can be infiltrated to produce recombinant proteins.

Theoretically, an alternative to transient viral vector expression would be to obtain transgenic plants that express viral vectors from constitutively or inducible plant promoter vectors. The first step in this direction was the expression of PVX-based insertion and replacement vectors in *N. tabacum* plants under the transcriptional regulation of a 35S *Cauliflower mosaic virus* promoter (Angell and Baulcombe 1997). This initial attempt to stably express PVX vectors in transgenic plants was unsuccessful because of the negative effects of VIGS, resulting in very low levels of PVX vector expression (Angell and Baulcombe 1997). In subsequent years, the advance in the understanding of viral post-transcriptional gene silencing (PTGS) suppressor proteins led to the development of a system, named "amplicon plus system", that consists in obtaining transgenic plants for PVX vectors and a mutant version of the *Tobacco etch virus* helper component-protease (TEV HC-Pro), a PTGS suppressor protein (Mallory *et al.* 2002). Using this strategy it was possible to express β-glucuronidase (GUS) protein to 3% of total protein from a PVX-replacement vector, while the PVX-insertion vector did not accumulate enough GUS protein. An important limitation of this system is that the strong PTGS suppressors known so far produce severe developmental problems in plants (infertility, dwarfism, tumours) when stably expressed (Chapman *et al.* 2004; Dunoyer *et al.* 2004). Thus, the use of this technique would greatly need the development of mutant versions of PTGS suppressors with attenuated deleterious effects in plants, a very difficult task to realize.

In principle, another way of evading the strong PTGS response of the plant would be to express the viral vector in a restricted period of time, especially after the host has reached adulthood, so as to avoid deleterious effects on plant development. The regulated expression of vectors needs the creation of inducible promoters that can be tightly controlled, something inherently difficult. An example in this direction is the work of Zhang and Mason (2006), who developed an inducible system combining a geminivirus *Bean Yellow Dwarf Virus* (BeYDV) replicon and a viral replication initiation protein (Rep) whose expression is controlled by an alcohol-inducible promoter. The BeYDV replicon consists in an expression cassette that expresses foreign proteins from the 35S *Cauliflower mosaic virus* promoter flanked by *cis*-acting DNA elements recognized by the Rep protein. When Rep expression is induced by ethanol, the BeYDV replicon is released from the stably integrated T-DNA and is episomally replicated to high copy numbers. This viral replicon was used to express GFP and the *Norwak virus* capsid protein in a tobacco cell line and in potato leaves resulting in an enhancement of transgene expression levels in induced lines, although low level of expression was also observed in non-induced plants (Zhang and Mason 2006).

A strategy based on induction by estrogen analogues was designed by Zuo *et al.* (2000). In this work, the authors created a chimeric transcription factor (XVE) containing the DNA-binding domain of the bacterial protein LexA, the activation domain of the viral VP16 protein and the ligand binding domain of the mammalian estrogen receptor. The transactivating activity of the XVE chimeric factor, which is expressed from a constitutive promoter, is tightly dependent on the presence of estrogen analogues, like estradiol and

tamoxifen. The GFP reporter, in its turn, was put under the control of LexA binding sites and thus under the regulation of estrogen analogues. Based on this system, Dohi *et al.* (2006) created a construct in which the whole TMV-based vector was put under direct control of the estrogen-inducible promoter and used to express GFP in tobacco BY-12 cell lines, obtaining high levels of GFP expression after the system was induced (10% of total soluble protein) and undetectable levels of expression in uninduced cells. Thus, this system could in principle be used to regulate viral vectors in transgenic plants for the expression of antigens.

To increase the "tightness" of the estrogen inducible system, Chua's group made use of the Cre/LoxP recombination system widely used in mammalian cells (CLX system, Guo *et al.* 2003). In this work, the transcription unit of a silencing hairpin was separated from its promoter by a DNA segment flanked by loxP sites. The Cre recombinase, in its turn, is controlled by a promoter activated by estrogen analogues like tamoxifen. Thus, when the plant is exposed to tamoxifen, the Cre recombinase is expressed, excises the loxP-flanked region that separates the transgene from the promoter and allows the expression of the transgene. This system was successfully used to avoid post-transcriptional silencing in non-induced transgenic plants that express a silencing hairpin, a strong PTGS inducer (Guo *et al.* 2003). The system was tightly controlled, and no leaky expression was observed. Our group has attempted to develop an inducible viral vector based on the one designed by Guo *et al.* We reasoned that its properties could be used to regulate the expression of the PVX vector avoiding any expression in the absence of inducer, overcoming in this way the plant PTGS response during the development of the transgenic plants. Unfortunately, all PVX tobacco transgenic lines we obtained showed expression of the PVX vector in the absence of estrogen, this basal expression of the vector was relatively high and maintained over three generations (Bey 2010). Our experience highlights the intrinsic difficulties in obtaining tightly controlled transgene expression systems.

CONCLUSIONS

Expression of antigens for vaccine production in plants offer several advantages over conventional methods, including scalability, lower production costs and human safety. In this context, viral vectors offer the advantages of speed of development and ease of manipulation, as well as enhanced antigenic capacity when antigens are incorporated into the viral capsid. The challenges ahead include the creation of better vectors capable of circumventing the silencing responses of the host plant and improved induction systems, as well as reliable and scalable ways of introducing the vector into host plants. Argentina has a tradition of being receptive to technological innovations in the agricultural field and a need for new vaccines for animal and human use, making it a fertile ground for the development and employment of new plant-based expression systems.

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