REVIEW

Plant viruses as scaffolds for the presentation of vaccine epitopes

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Abstract

Within the last two decades, plant viral vectors have emerged as an excellent tool for the expression of foreign peptides and proteins. Virus particles carrying foreign antigenic epitopes present some interesting advantages for vaccine design and other applications. This review covers recent advances in the use of some typical plant viruses with helical particles that present heterologous peptides with particular emphasis on particles derived from the *Potato virus X* (PVX) and its uses.

Additional key words: transient expression, plant viral expression vectors.

Introduction

Plant viruses for the presentation of epitopes: Plant viruses are considered potentially safe platforms for animal and human vaccine preparation, as well as delivery systems for therapeutic agents and contrast dyes in animal and human therapy (Jokerst and Gambhir 2011, Lico *et al.* 2013). The main advantages of plant viruses as vaccines are their biocompatibility, chemical and physical stability, non-infectivity and non-toxicity to animal and human cells, as well as the scalability of their expression in plants. To develop a vaccine, an antigen of interest is exposed on the surface of the virion with the cognate genetic information fused to the coat protein (CP) (Cañizares *et al.* 2005). Assembled virus particles thus display highly ordered, repetitively arrayed immunogenic peptides which are very effective in stimulating the immune system. These particles induce potent T-cell mediated immune responses by stimulating the dendritic cells. These displaying systems are sometimes called protein/peptide presentation systems, and those based on plant viruses are good candidates for the production of safe and inexpensive vaccines. This has particular relevance for the needs of developing countries (Hefferon 2013).

The use of plant viruses as carriers of foreign peptides is the subject of ongoing research. The sequence encoding the peptide is inserted at a suitable location within the CP gene as an in-frame fusion so that the peptide is displayed on the virus surface. To realize the full potential of plant expression platform, the preferred administration route would be nonparenteral so that minimal product purification would be required. If a purified product is required, the chimeric virus particles can be extracted from the infected plant material using standardized protocols employed for plant virus isolation. Alternatively, the foreign peptides can be attached through chemical linkage. Besides modification of the outer surface of virus particle, there is a growing interest in modification of the inner cavity of the virion, especially of spherical viruses, rendering these particles self-assembling supramolecular structures (reviewed by Saunders and Lomonossoff 2013). Establishment of a plant virus based epitope presentation platform involves

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Submitted 26 February 2016, *last revision* 31 May 2016, *accepted* 2 June 2016.

Abbreviations: aa - amino acid; AlMV - *Alfalfa mosaic virus*; CP - coat protein; CPMV - *Cowpea mosaic virus*; GFP - green fluorescent protein; GUS - β-glucuronidase; HC-Pro - helper component proteinase; HPV - *Human papillomavirus*; HR - hypersensitive response; mAbs - monoclonal antibodies; MP - movement protein; ORF - open reading frame; PPV - *Plum pox virus*; PVA - *Potato virus A*; PVX - *Potato virus X*; PVY - *Potato virus Y*; RdRp - viral replicase; ssRNA - single stranded RNA; TMV - *Tobacco mosaic virus*; TRV - *Tobacco rattle virus*; TSP - total soluble protein; TuMV - *Turnip mosaic virus*; VLP - virus like particles.

Acknowledgements: This research was supported by the Grant Nos. GA15-10768S and P501/12/1761 of the Czech Science Foundation, CZ.2. 16/3.1.00/24014 from the European Regional Development Fund, Operational Programme Prague-Competitiveness.

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the development of a viral expression vector, choosing a suitable plant host, infecting the plant host, purification and downstream processing of the desirable product.

Plant viral vectors: An alternative to stable plant genetic transformation, is expression of heterologous proteins or peptides using plant viral vectors. The main advantages of this method are that viral genomes are relatively small and easy to manipulate at a molecular level, infection of plants with viruses is simpler and more rapid than the regeneration of stably transformed plants, and the sequence inserted into a viral vector will be greatly amplified resulting in much higher expression of the desirable product than is usually achieved through plant transformation. However, there are some disadvantages to this: the foreign gene is not heritable, there are limitations to the size and the context of the sequences that can be expressed as opposed to the genetically stable expression, and concerns about the release of modified viruses into the environment. The use of the plant virus approach relies on the fact that viruses can infect the plant, producing either local or systemic infection, generating multiple genome copies (Rosales-Mendoza 2014).

Originally, the viral replication was initiated either by *in vitro* transfection of infectious nucleic acid of the viral vector or, preferably, with assembled viral particles. The infection process can be performed on a large scale by spraying host plants in the field with a mixture of viral particles and an abrasive such as carborundum. Depending on the efficiency of the viral vector and the ability of the virus to move systemically, one to three weeks are required for most of the tissues of the transfected plants to become infected (Gleba *et al.* 2005a).

Another technical breakthrough was the use of *Agrobacterium tumefaciens* to promote viral infection of plants (Grimsley *et al.* 1986, Turpen *et al.* 1993, Azhakanandam *et al.* 2007). The use of *Agrobacterium tumefaciens*, a procedure known as ‘‘agroinfection”, allowed the use of viruses that in nature are not mechanically transmissible. Vacuum infiltration of plants is robust and can be scaled-up for commercial scale manufacture of pharmaceutical proteins. This also enables agroinfiltration of plant species that are not amenable to syringe infiltration such as lettuce and *Arabidopsis* (Leuzinger *et al.* 2013).

Viral vectors are commonly divided into complete viral vectors, deconstructed vector systems, gene substitution vectors, gene insertion vectors and peptide display vectors (Fig. 1).

Complete viral vectors are essentially fully functional viruses that are engineered to express selected genes of interest, in addition to all of the wild type virus genes. Although the expression levels usually reached using this full virus strategy are considered acceptable [up to 10 % of total soluble protein (TSP) of heterologous protein or over 1 g of recombinant protein per kg of biomass], first generation vectors had limitations, namely the inability to produce hetero-oligomeric proteins and size limitation in terms of length of protein of interest (proteins larger than 30 kDa were poorly expressed) (Salazar-González *et al.* 2015).

Deconstructed vectors are a new generation of viral expression system (Gleba *et al.* 2004). Their development was driven by the understanding that: *1*) not all viral components are essential or beneficial for an expression vector and *2*) viruses can be broken down into different genomic elements that would still operate together in the infection process as wild-type multipartite viruses. Moreover, agroinfection provided the technical possibility to co-deliver multiple different components in a single plant. Hence, in modular systems, viral components are separated into distinct portions and inserted into binary vectors contained in *Agrobacterium* (Lico *et al.* 2008). Currently, one of the most efficient and broadly used vectors is the deconstructed system based on the *Tobacco mosaic virus* (TMV) and developed by *Icon Genetics* (Halle, Germany). Deconstructed vectors rely on an integrated system that possesses the minimum viral elements required for viral replication, while some other functions such as DNA delivery are provided by non-viral elements (such as replicon formation *via* T-DNA-delivery by *A. tumefaciens*). With this approach, the production of functional infectious viral particles is avoided and thus the virus does not move systemically to newly grown leaves, the lack of systemic viral infection does not reduce production and also the risk of unintended virus spread is minimized (Gleba *et al.* 2005a). The deconstructed viral vectors typically provide higher production yields than those attained with first generation vectors, with maximum production yields of 50 % total soluble proteins or 5 g per kg of biomass in 4 - 15 d. Another advantage of the deconstructed viral vectors is the size of the target sequence that can be maintained. This may be up to 2 kb inserts or up to 80 kDa proteins. The factors influencing the efficiency of these expression systems include the virus on which the system is based, the size of the target gene, the host plant and the agrobacteria density in the agroinfiltration step (Mortimer *et al.* 2015, Salazar-González *et al.* 2015).

Gene substitution or replacement vectors are based on the exchange of an endogenous viral sequence with a heterologous gene of interest. Gene insertion vectors consist of complete functional viruses with the addition of an extra open reading frame (ORF) for the target protein (Lico *et al.* 2008).

Many plant expression vectors are available. Most derive from positive single-stranded RNA [(+) ssRNA] plant viruses. The first plant virus to be developed as an epitope presentation system was the *Cowpea mosaic virus* (CPMV; Porta *et al.* 1994) followed shortly later by TMV (Fitchen *et al.* 1995). To date, the genomes of plant viruses belonging to at least eight different genera of RNA viruses have been modified into vectors (Mortimer e*t al.* 2015), including the tobamoviruses, tombusviruses, bromoviruses, potexviruses, comoviruses, benyviruses, potyviruses, and cucomoviruses. In addition, virus

vectors have also been developed using the small circular ssDNA genomes of at least 12 members of the *Geminiviridae* family (Hefferon 2014, Rybicki and Martin 2014). Among the most exploited viral vectors are the vectors based on the TMV, *Potato virus X* (PVX), CPMV, *Alfalfa mosaic virus* (AlMV), and *Plum pox virus* (PPV) (Hefferon 2012).

Chemical linking of peptides to virus particles: As an alternative to genetic fusions of peptides, the virus particles could be decorated with peptides or even complete protein antigens through chemical crosslinkers that bind specifically to either natural or engineered reactive groups on the virion surface. This approach overcomes several drawbacks of direct genetic fusion of foreign epitope to viral particle. Typically, a wild type virus or a virus with engineered reactive amino acid residue is used, thus overcoming problems with expression, solubility, and particle assembly. Further, several different epitopes can be presented in various stochiometric ratios and non-proteinaceous chemical moieties can also be attached when needed. With this approach, there is apparently no limit to antigen size and a variety of different epitopes can be exposed on a single viral particle. However, the drawback of this approach is that the virus particle always has to be purified prior to chemical linkage. Several helical plant viruses have been used for the surface display of exogenous peptides using chemical linkage. Recently, human epidermal growth factor receptor 2 (HER2) B-cell epitope was linked to a PVX and used to overcome immunological tolerance to HER2. The HER2 derived P4 peptide was conjugated to the surface lysines on PVX coat proteins *via* heterofunctional *N*-hydroxysuccinimide-PEG4-maleimide linker (Shukla *et al.* 2014). PVX was also used as a carrier of a non-covalently linked weak idiotypic tumor antigen, the tumor-specific Ig expressed by a murine B cell lymphoma (Jobsri *et al.* 2015).

Plant hosts: An integral part of the successful platform for molecular pharming using chimeric viruses is the selection of suitable plant host. The choice of host plant depends on a broad range of criteria including the nature of the protein, post-translational modifications, secondary metabolites, biomass yield, scale-up of production and maintenance costs, span of production cycles, and the downstream processing requirements. A wide range of plant crops has been tested for molecular farming purposes, including leafy crops, cereal and legume seeds, oil crops, plant cell suspensions, hairy roots, and microalgae. Leafy crops are helpful in terms of biomass yield and high soluble protein content (Conley *et al.* 2011). However, there is a major problem of instability of the expressed proteins in leaves due to proteolytic degradation with aging of the leaves. In fact, the instability of proteins present in leaf cells, and also in cells of the other plant tissues, may start as early as during the translation of the foreign proteins, which have a natural tendency to structural heterogeneity in a heterologous environment (Streatfield *et al.* 2007).

Plant viral vectors are currently available for recombinant protein expression in a wide range of host plants including *Nicotiana benthamiana* (Dolja *et al.* 1992), tobacco (Hagiwara *et al.* 1999), squash (Chen *at al.* 2005), cucumber (Hsu *et al.* 2004), wheat (Choi *et al.* 2000), barley (French *et al.* 1986, Haupt *et al.* 2001, Lacomme *et al.* 2003), cowpea (Allison *et al.* 1988, Gopinath *et al.* 2000), *Nicotiana clevelandii* (Fernández-Fernández *et al.* 2001), *Chenopodium quinoa*, *Arabidopsis thaliana* (Turnage *et al.* 2002), soybean (Zhang and Ghabrial 2006a), *Pisum sativum* (Constantin *et al.* 2004), turnip (Brisson *et al.* 1984), spinach (Yusibov *et al.* 2002), and *Brasicca rapa* (Hoffmeisterová *et al.* 2008).

Tobacco and related *Nicotiana* species are currently the most common host plants for the generation of plant-made pharmaceutical proteins. The major advantage of tobacco over other herbaceous hosts is its great biomass yield, well-established technology for gene transfer and expression, year-round growth and harvesting, and the existence of large-scale infrastructure for processing. Furthermore, tobacco as a non-food and non-feed crop possesses little risk of contamination of either food or the food chain (Makhzoum *et al.* 2014). However the downstream processing of target plant-made pharmaceutical proteins from tobacco is hindered by potential technical and regulatory difficulties due to the presence of high content of phenolics and toxic alkaloids.

Alternative leafy crops such as lettuce (*Lactuca sativa*) are also being investigated as a production host for plant-made pharmaceutical proteins. Lettuce grows quickly and produces low content of secondary metabolites. Lai *et al.* (2012) showed that a geminiviral replicon system based on the *Bean yellow dwarf virus* permits high expression in lettuce of VLPs derived from the Norwalk virus capsid protein and therapeutic monoclonal antibodies (mAbs) against Ebola and West Nile viruses. Furthermore, the human growth hormone (hGH) was transiently expressed in tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*) and lettuce leaves (Sohi *et al.* 2005).

Besides whole plants, plant viral vectors have also been deployed in plant tissue cultures or suspension cultures (Fischer *et al*. 2012). In fact, the first plant that was pharmaceutically approved for human use, Elelyso, is produced using carrot cell culture (Grabowski *et al*. 2014). Larsen and Curtis (2012) modified replicating vectors derived from PVX and *Tobacco rattle virus* (TRV). In cell suspensions, a minimal PVX vector retaining only the viral RNA polymerase gene yielded 6.6-fold more β-glucuronidase (GUS) than an analogous full-length PVX vector. Transient co-expression of the minimal PVX vector with P19 of *Tomato bushy stunt virus* or HC-Pro of *Tobacco etch virus* aimed at suppressing post-transcriptional gene silencing of the expressing viral vector, increased GUS expression by 44 and 83 %, respectively. In hairy roots, a TRV vector capable of systemic movement increased GUS

accumulation 150-fold compared to the PVX vector. Histochemical staining for GUS in TRV-infected hairy roots revealed the capacity for achieving even higher productivity per unit biomass. The content achieved in *N. benthamiana* hairy roots were 1 - 2 orders of magnitude greater than in suspended cells of the same species, reaching 1 - 2 mg g^{-1}(d.m.) or 20 - 28 % of TSP. However, protein degradation in cell suspensions is recognized as playing an important negative role, occasionally limiting the overall yields to less than 1 % TSP (Doran 2006).

Hairy roots newly infected with TMV were cultured for 35 d, then subcultured to generate root cultures with established viral infections (Shadwick and Doran 2007). In other studies, *N. benthamiana* hairy roots were generated from the leaves of plants that had been previously infected with a TMV-based viral vector for expression of green fluorescent protein (GFP) (Skarjinskaia *et al.* 2008). The hairy roots expressed GFP at concentrations of 50 - 120 µg g^{-1}(f.m.) and maintained their ability to produce foreign protein over a 3-year period.

Recently, Rademacher (2014) presented a new ”cell pack” platform. The platform is based on agroinfection of plant cell suspension culture material (*e.g.*, tobacco BY-2 cells) in the form of medium deprived, porous structured multilayer cell pack also called artificial leaf. This technology has great potential for industrial scale transient protein expression using viral vectors.

Downstream processing and the economy scale: Typically, 80 % of the total production costs of bio-pharmaceutical protein is in downstream processing (Fischer *et al.* 2013, Walwyn *et al.* 2014). While the protocols are often product specific, for the isolation of chimeric virus particles, a generic protocol could be used.

Established laboratory protocols for the purification of virus particles frequently contain ultracentrifugation step(s). This is, however, not practical on a large scale when several tones of plant biomass have to be processed at one time. Thus, attempts were made to adapt these protocols for large scale use. One such example is the process where the crude plant extract is first briefly heated to 45 - 50 °C to precipitate soluble plant proteins and the virus particles are then concentrated *via* ultrafiltration and finally precipitated by salt and polyethylene glycol (PEG 8000). The authors report yields of 0.6 - 1 kg of TMV particles at 95 % purity from one acre (4047 m^2) (Garger *et al*. 2001). It is interesting that this generic protocol could be used for about 40 % of chimeric constructs while the remaining chimeras could be purified with slight modifications and pH adjustments. Although the final chimeric virus is nonreplicating in mammals, it could still be regulated as a plant pathogen. Thus, additional steps might be needed to deactivate the viral RNA (Smith *et al.* 2006).

It is generally recognized that the yield of expressed protein varies more significantly among multiple batches of transiently expressed proteins than between batches of transgenic plants. Unfortunately, current cGMP requirements demand high reproducibility and consistency (O’Neill *et al.* 2008). The study of Buyel *et al.* (2013) describes a model based on transient expression of anti HIV monoclonal antibody using a non-replicating vector in *N. tabacum*. They found that by careful control of the growing conditions, the variation could be reduced to similar levels to those observed in transgenic systems. Interestingly, younger, rapidly growing plants with intensive protein synthesis are more suitable for protein expression and downstream processing than slightly older plants having a twice as high biomass content. The authors also observed a dramatic (several fold) effect of temperature on levels of expressed protein. There are only few studies on the economic viability of the whole production process. One such study, which is also applicable to the isolation of chimeric plant virus particles, describes the transient production of peroxidase enzyme (Walwyn *et al*. 2014). A still substantial percentage (59 %) of the total costs includes the high capital investment needed for, *e.g.*, greenhouse and processing facilities. Raw materials on the other hand represented only a tiny fraction of the total costs (4 %). Using the peroxidase model, the authors concluded that the transient plant expression would be competitive only when considerable economies of scale are reached, in this case annual production of 5 kg of purified enzyme. Significant improvements could be made by increasing the plant biomass productivity of greenhouses and/or the protein expression.

Plant viruses as viral vectors

***Tobacco mosaic virus*:** TMV is one of the best studied viruses. It has the (+) ssRNA genome of 6,4 kb coding for viral replicase (RdRp), movement protein (MP), and coat protein (CP). The viral particle contains capped non-polyadenylated RNA and more than 2 100 copies of the viral CP. The particles are rigid rods 300 nm in length and 18 nm in diameter. The virus can accumulate to extremely high levels in inoculated plants reaching 10 g per kg of leaf biomass. TMV has also a wide host range as it is known to infect about 200 plant species. Its ability to accumulate to extremely high concentrations is widely used for the construction of viral expression vectors (Gleba *et al.* 2005b). TMV virions are highly stable. They can withstand heating to 50 °C and even retain infectivity following exposure to enzymes in the human or animal digestive system (Zhang *et al.* 2006b). The conditions for polymerization and assembly into particles have been thoroughly investigated (for review see Butler 1999). Moreover the 3D structure of the viral coat has been determined to atomic resolution and thus simplifying site

directed mutagenesis and epitope presentation (Bhyravbhatla *et al.* 1998).

Haynes *et al.* (1986) fused TMV CP to poliovirus VP1 epitope and the construct was expressed in *E. coli* to obtain neutralizing antibodies in immunized rats. Shortly after the first infectious clones of TMV were produced (Dawson *et al.* 1986), they were used to create chimeric virus particles carrying foreign epitopes. Thus, TMV was among the first plant viruses to be used to display immunogenic epitopes *in planta*. Turpen *et al.* (1995) reported successful display of duplicated 6 amino acid (aa) malaria epitope while the group from the Scripps Research Institute group (Fitchen *et al.* 1995) used chimeric TMV carrying 13 aa epitope from ZP3 protein of murine zona pellucida to mount an immune reaction with contraceptive effect in mice. This was a particularly interesting result since it showed that short peptides presented on plant virus particle can disrupt immune tolerance to self antigens.

Another important concept was established by Koo *et al.* (1999). These authors successfully used chimeric TMV particles to stimulate mucosal immunity. In this study, the 5B19 epitope (10 aa long peptide) from the spike protein of *Murine hepatitis virus* (MHV) was inserted between amino acid residues 154 and 155. After purification from *N. tabacuum* cv. Xanthi, recombinant virus was intranasally administered to mice that developed serum IgG and IgA specific for the 5B19 epitope. Furthermore, intranasally immunized mice were protected against MHV infection after challenge with a lethal dose of MHV. Another important step in achieving mucosal immunity was described by Wu *et al.* (2003) who demonstrated effective protection against a foot-and-mouth disease virus (FMDV) challenge in guinea pigs vaccinated by oral administration of TMV virions carrying FMDV epitopes. These results showed the potential of chimeric viral particles to induce relevant mucosal immune responses by non-parenteral application/administration, which would substantially reduce or even eliminate the purification costs.

While the initial successes with display of various epitopes was then replicated by other groups, it was also apparent from early on, that presentation of some epitopes leads to issues with low expression, low solubility, and/or tendency to initiate a hypersensitive response (HR) in host plants (Fitchen *et al.* 1995). Bendahmane *et al.* (1999) showed that adding more compensatory acidic amino acid residues that brought the pI of chimeric virus near to the wild type (wt) CP, reduced or eliminated the ability of virus to induce HR response and cell death.

For some time, it was believed that the presentation of longer epitopes (*i.e.*, more than about 25 aa) on the TMV CP was impossible. However, Werner *et al.* (2006) showed that even longer protein domains connected to the C-terminus of TVCV tobamovirus *via* 15 aa linker could be efficiently expressed in plants and assembled into particles.

Another interesting application of TMV particles as scaffolds was using a mutant TMV that carried reactive lysine residue near its N-terminus. This TMV mutant was derived from a combinational library and selected for similar accumulation and solubility as wild type TMV. Several cytotoxic T lymphocyte epitopes and arginine-glycine-aspartic acid uptake motives were then chemically conjugated *via* bifunctional linker. The conjugated particles showed 1000-fold improved efficacy over unconjugated peptide (McCormick *et al.* 2006b).

Potyvirus particles: Potyviruses are flexuous, non-enveloped, rod-shaped particles 680 - 900 nm long and 11 - 15 nm wide. They are composed of (+)ssRNA, about 10 kb long, surrounded by about 2 000 copies of CP subunits. The RNA genome carries a VPg (viral protein genome-linked) covalently bound to its 5′ end, and a poly(A) tail at its 3′ end. The genome contains a single long open reading frame (ORF) translated into a large 340 - 370 kDa polyprotein that is co- and/or post-translationally cleaved to produce cleavage intermediates or the final protein products (Urcuqui-Inchima *et al.* 2001). The proteolytic processing strategy of potyviruses ensures that a foreign protein is synthesized as part of the viral polyprotein and thus is produced in equimolar amounts with all viral proteins (Scholthof *et al.* 1996, Spall *et al.* 1997). Expression of foreign genes by potyviruses has been demonstrated in *Tobacco etch virus* (TEV) (Dolja *et al.* 1992), *Plum pox virus* (PPV) (Guo *et al.* 1998, Fernández-Fernández *et al.* 1998, 2001), *Lettuce mosaic virus* (LMV) (Choi *et al.* 2000, German-Retana *et al.* 2000), *Zucchini yellow mosaic virus* (Arazi *et al.* 2001, 2002, Hosseini *et al.* 2012), and more recently in *Turnip mosaic virus* (TuMV, Touriño *et al.* 2008). An interesting application of potyviral nanoparticle (VNPs) for non-vaccine purposes was devised by Ponz *et al.* (2013), who used TuMV particles for antibody detection with improved sensitivity over natural HSP60 antigen. Using the same system, epitopes from the *Maedi-Visna virus* were expressed on TuMV VNPs and used for antibody detection in sheep sera. A 20-amino acid peptide derived from the human vascular endothelial growth factor receptor 3 (VEGFR-3) was fused to the N-terminus of TuMV CP and displayed on the surface of viral particles in two different host plants (*Arabidospsis thaliana* and *Brassica juncea*) (Sánchez *et al.* 2013). Manuel-Cabrera *et al.* (2012) evaluated the availability of amino groups on the surfaces of TEV particles and the immune response to TEV.

***Potato virus A*:** Another attempt was made by Cerovska *et al.* (2008). These authors aimed to express the construct, which was derived from HPV16 E7 peptide (aa 44 - 60) used for DNA (Pokorná *et al.* 2005) and the epitope derived from the minor capsid protein HPV-16 L2 expressed as N-terminal fusion with a CP of *Potato virus A* (PVA). This construct was expressed in *N. benthamiana* (the yield obtained was 28 to 43 μg g^{-1}(leaf f.m.) and in *Brassica rapa* (Hoffmeisterová *et al.* 2008). To increase the content of expressed protein,

transgenic *N. benthamiana* plants expressing PVA *HC-Pro* gene and synergistic infection of host plants with *Potato virus Y* (PVY) was tested (Cerovska *et al.* 2008).

***Potato virus X*:** PVX is a member of the family *Alphaflexiviridae* in the genus *Potexvirus*. It infects several solanaceous crops including potato, tomato, and tobacco. Symptoms produced by PVX are variable, depending on the strain and host plant. In general, plants often do not exhibit symptoms, but the virus can cause symptoms of mild mottling, chlorosis, mosaic, or decreased leaf size. It can be mechanically transmitted. PVX has a monopartite (+)ssRNA of approximately 6.4 kb (Skryabin *et al*. 1988, Tollin and Wilson 1988). The genome organization has been shown to contain five ORFs (Morozov *et al*. 1991). The CP and triple gene block (TGB) proteins are involved in cell-to-cell and long-distance movement of viral particles *via* the plasmodesmata (Santa Cruz *et al.* 1998, Voinnet *et al*. 2000, Fedorkin *et al*. 2001, Samuels *et al*. 2007).

PVX has significant potential for protein expression in biotechnology. It has been shown to be an ideal, highly ordered, multivalent scaffold for use to this end (Brennan *et al.* 1999, Marusic *et al.* 2001). In plants, PVX has been utilized as a full-length expression vector capable of infecting distal tissues as well as a deconstructed vector lacking viral genes essential for local or systemic movement. Several strategies for developing transient expression vectors derived from PVX have been invented (Salazar-Gonzáles *et al.* 2014). Vectors derived from PVX are based on the use of *1*) a duplicated sub-genomic promoter (Baulcombe *et al.* 1995), *2*) a single transcription unit encoding for CP and the desired antigen separated by the foot-and-mouth disease virus (FMDV) 2A catalytic peptide (Santa Cruz *et al.* 1996), *3*) a bicistronic mRNA containing internal ribosome entry site (Toth *et al.* 2001), *4*) a fusion of foreign coding sequences to the N-terminus of PVX-CP (Uhde *et al.* 2005, Uhde-Holzem *et al*. 2007), *5*) a substitution of TGBp and CP genes by GFP to reduce the size of the vector (Komarova *et al.* 2006), or *6*) a hybrid viral vector (Tyulkina *et al.* 2011). Very recently, because of the genetic instability due to promoter duplication, the PVX-based vectors were stabilized by combining heterologous subgenomic promoter-like sequences with an N-terminal PVX CP deletion (Dickmeis *et al.* 2014). Moreover, a new PVX-based vector containing two additional subgenomic promoters can be utilized for simultaneous expression of two foreign proteins (Wang *et al* 2014).

Use of PVX for vaccination against *Human papillomavirus*: *Human papillomavirus* (HPV) constitutes a large group of DNA viruses of about 140 types belonging to family *Papillomaviridae*. HPVs represent one of the most common sexually transmitted infections worldwide. Among high-risk HPVs, the most common genotypes are HPV-16 and 18, together responsible for approximately 70 % of cervical cancers. HPV-16 alone is particularly important in light of the fact that it causes 50 % of cervical cancers (Zur Haussen 2002, Montgomery and Bloch 2010).

A prophylactic vaccine preventing persistent HPV infection could substantially reduce the incidence of cervical cancer. This type of HPV vaccines is based on the viral capsid proteins L1 and L2 and it induces neutralizing antibodies against virions. Several bivalent to 9-valent prophylactic vaccines based on recombinant L1 VLPs are on the market (Edney 2012). Vaccines containing the minor L2 protein are being investigated because of their potential for cross-protective efficacy (Dochez *et al.* 2014, Tyler *et al.* 2014).

Despite the great efficacy of prophylactic HPV vaccines, they do not have any therapeutic effects against preexisting infection. To cure HPV-induced premalignant or malignant lesions, therapeutic vaccines should elicit cellular immune responses capable of eliminating HPV-infected cells. Viral oncoproteins E6 and E7 proteins thus present ideal target antigens for the development of therapeutic vaccines (Hung *et al.* 2007). Experimental vaccines based on the high-risk HPV oncogenes E6 and E7 have demonstrated efficacy in animal models. However, while the major need for these vaccines would be in developing countries, the costs of these vaccines might make them prohibitively expensive (Rybicki 2014). One possible approach to reduce the vaccination costs is to use plant-based production platforms (Giorgi *et al.* 2010, Rybicki 2010).

One of the first promising studies in this field was the use of a PVX-derived expression vector to express E7 antigen from HPV. The soluble HPV 16 E7 protein was produced with a PVX based vector in both inoculated and systemic leaves in *N. benthamiana* plants. E7-containing foliar extract induces a humoral and also cell-mediated immune response in mice. The plant extract acts *per se* as a potent adjuvant (Franconi *et al.* 2002).

PVX N-terminal fusions: Mutational analysis of PVX CP revealed that the central and the C-terminal part of PVX CP are required for systemic infection, whereas the N-terminal part is not necessary for virion formation. However, N-terminus may influence intra- or inter-molecular interactions in the virus particle (Chapman *et al.* 1992). Recently, the PVX CP N-terminal segment was found to be important for virion assembly (Lukashina *et al*. 2012). The N-terminus of PVX CP was shown to be highly immunogenic (Sõber *et al.* 1988). It has been demonstrated that when the N-terminus of PVX CP, rich in serine/threonine aa, is phosphorylated, it induces conformational changes in PVX CP and the RNA in PVX virions become translationally activated (Atabekov *et al*. 2001). Moreover, the N-terminus is also proposed to be glycosylated and this is important for binding water molecules around the virion probably for maintaining the virion surface structure (Baratova *et al.* 2004). The virus particle assembly is essential for cell to cell and long-distance transfer where the N-terminus plays a pivotal role (Betti *et al.* 2012).

There have been a large number of attempts to

produce vaccines using N-terminal fusions of antigens with PVX CP, which were able to form chimeric virus particles. As examples, Marusic *et al.* (2001) discussed the expression of a neutralizing epitope from human immunodeficiency virus type 1 (HIV-1) on the surface of a plant virus and its immunogenicity. In another study, rotavirus major inner capsid protein VP6 was expressed in *N. benthamiana* plants either as soluble protein or as a fusion with PVX CP. The fusion protein yields both VP6-coated PVX rods and self-assembled VP6-VLPs (O´Brien *et al.* 2000). In addition, the genetic stability of a recombinant PVX vector presenting multiple epitopes of *Beet necrotic yellow vein virus* (BNYVV) was investigated. PVX particles displaying ep4 alone, ep6 alone, or both epitopes were formed (Uhde *et al*. 2005). Marconi *et al.* (2006) reported *in planta* production of peptides of the classical swine fever virus (CSFV) E2 glycoprotein fused to the PVX CP. Partially purified virions induce anti-E2 antibodies in immunized rabbits. Lico *et al.* (2009) expressed PVX particles displaying an epitope of influenza A virus nucleoprotein (NP) in *N. benthamiana*. Furthermore, the E7 oncoprotein was fused to PVX CP and expressed it in tobacco chloroplasts with higher yields than E7 oncoprotein alone (Morgenfeld *et al*. (2009). Chimeric PVX particles displaying the hepatitis C virus hypervariable region 1 peptide R9 derived from envelope protein E2 were expressed in *N. benthamiana* (Uhde-Holzem *et al*. 2010). Cerovska *et al.* (2012) expressed the construct derived from minor capsid protein HPV-16 L2 in plants resulting in immunoreactive displayed epitopes, where the genetically modified virus is distributed systemically and the yield is 17 mg of recombinant protein per 100 g of infected tissue, which is similar to the yield of unmodified wild type virus.

C-terminal fusions of PVX: To display heterologous proteins/peptides on the surface of the virion, foreign coding sequences are often added only to the N-terminus of PVX CP. Such fusions do not usually impair the replication or assembly of the recombinant virus and thus allow expression at high levels. However, the generation of chimeric virus particles strongly depends on the isoelectric point and the tryptophan content of the inserted peptide and the genetic stability of the foreign aa sequence depends on the presence of serine and threonine residues in the peptide (Lico *et al*. 2006, Uhde-Holzem *et al.* 2007). Some fusions are therefore unstable or prone to mutations. This is also the case for the mutagenized form of HPV-16 E6 oncoprotein (E6GT) fused to the N-terminus of PVX CP. The nucleotide sequence of E6GT mutates when present in plants (Cerovska *et al.* 2013). Other surface-exposed sites, such as the C-terminus, are therefore required to display desired proteins/peptides on the surface of the virion.

The deletion of the 10/18 C-terminal aa of PVX CP results in the loss of virion ability to bind TGBp1 and to be translationally activated, suggesting that the C-terminus is oriented to the surface of the virion 5´-end. These virions were converted into a translatable form by *in situ* CP phosphorylation. PVX CP mutants with a deletion longer than 18 aa are not able to produce virus like particles (VLPs) on incubation with RNA (Zayakina *et al*. 2008). Moreover, a deletion of 18 C-terminal aa of PVX CP results in loss of movement function which can be complemented *in trans* by the CPs of other filamentous viruses (Fedorkin *et al.* 2000 and 2001). In addition, the PVX 3´non-translated region contains structures important for both plus- and minus-strand RNA synthesis (Pillai-Nair *et al.* 2003, Hu *et al.* 2007, Verchot-Lubicz *et al.* 2007).

For the first time, PVX CP, C-terminus was used for protein expression in plants. Mutagenized E7 oncoprotein (E7ggg) from HPV-16 was fused to the C-terminus of PVX CP (Plchova *et al.* 2011. The same approach was used for expression of HPV-16 L2 (aa 108 - 120; $L2_{108\text{-}120}$) epitope on the C-terminus of PVX CP (Hoffmeisterova *et al.* 2012), however it seems that this C-terminal modification of the PVX CP makes virion assembly *in vivo* impossible (Cerovska *et al.* 2012). To display another HPV-16 antigen on PVX particles, mutagenized oncoprotein E6 (E6GT) was transiently expressed as C-terminal fusion with PVX CP using the same system previously designed for E7ggg (Plchova *et al.* 2011). The chimeric protein accumulates in inoculated leaves of *N. benthamiana* plants and forms viral particles. However, these recombinant viruses are not able to spread systemically (Cerovska *et al.* 2013). The possibility of displaying antigens as insertions into putative surface-exposed loops was therefore investigated.

Internal loops of PVX: The main obstacle for peptide presentation in the surface-loops of PVX virus particles is still the lack of information on the high-resolution structure for PVX. Using fiber diffraction, the surface of the PVX virion was described, showing that it has a helical structure with 8.9 subunits per helical turn and two sets of grooves – vertical and horizontal (Parker *et al.* 2002). Models for the tertiary structure of a PVX CP subunit within the virion have been proposed (Dobrov *et al.* 2007, Nemykh *et al.* 2007, 2008, Lukashina *et al*. 2009, 2012).

Although the model provides only limited accuracy, it was used as a template for designing new insertion sites in PVX CP for antigen presentation. For this purpose, epitopes from HPV were used. The HPV E7 epitope (aa 44 - 60) together with StrepII tag was inserted into four different putative surface exposed loops (Vaculik *et al*. 2015). The recombinant peptide located after aa 23 in the loop l2 in the modified virus infects plants systemically, expressed proteins assemble into viral particles and the epitopes are located on the particle surface. Here, it was shown that this peptide could be expressed together with the StrepII tag as an internal part of PVX CP. Some examples of epitopes and antigens fused to coat proteins of plant filamentous viruses are summarized in Table 1 Suppl. and 2 Suppl.

Conclusions

Since the first proof of concept studies describing plant viruses as a scaffold to present heterologous peptides in the mid 1990s, this field has seen enormous progress in encompassing the design of new improved viral vectors, strategies to present the heterologous peptide on viral particle, methods of initiating the viral infection cycle, biosafety, and also in potential applications of such chimeric particles. Progressively, we can observe a clear shift from fundamental academic research to the development of commercial products. We can see a number of novel plant derived products move through clinical trials and awaiting approval. Plant expression systems based on viral vectors, optimized and implemented on a commercial scale, show the greatest potential for offering biologically active products, also mammalian, insect, and yeast cell bioreactors. However, plant based products promise greater safety and they are potentially less expensive to manufacture. We hope that the inherent advantages of plants and plant viruses in particular will lead to novel applications in the future.

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