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Transient expression systems for plant-derived biopharmaceuticals

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In the molecular farming area, transient expression approaches for pharmaceutical proteins production, mainly recombinant monoclonal antibodies and vaccines, were developed almost two decades ago and, to date, these systems basically depend on *Agrobacterium*-mediated delivery and virus expression machinery. We survey here the current state-of-the-art of this research field. Several vectors have been designed on the basis of DNA- and RNA-based plant virus genomes and viral vectors are used both as single- and multicomponent expression systems in different combinations depending on the protein of interest. The obvious advantages of these systems are ease of manipulation, speed, low cost and high yield of proteins. In addition, *Agrobacterium*-mediated expression also allows the production in plants of complex proteins assembled from subunits. Currently, the transient expression methods are preferential over any other transgenic system for the exploitation of large and unrestricted numbers of plants in a contained environment. By designing optimal constructs and related means of delivery into plant cells, the overall technology plan considers scenarios that envisage high yield of bioproducts and ease in monitoring the whole spectrum of upstream production, before entering good manufacturing practice facilities. In this way, plant-derived bioproducts show promise of high competitiveness towards classical eukaryotic cell factory systems.

KEYWORDS: *Agrobacterium* • monoclonal antibodies • plant virus • transient expression • vaccine • virus-like particles • virus peptide display

Genes encoding vaccine proteins can be expressed in plant tissues exploiting different strategies for stable transgene expression, such as nuclear genomic integration [1] or expression from the plastid genome [2,3]. Transient gene expression provides a rapid alternative to the material- and time-consuming generation of stably transformed plants. When DNA is delivered into a plant cell, only a tiny proportion will become integrated into the host chromosomes and episomal DNA molecules can remain transcriptionally competent for several days. This transient expression does not depend on chromosomal integration and is not affected by position effects. Expression from extrachromosomal transgenes can be detected even 3 h after DNA delivery, reaches the maximum between 18 and 48 h, and persists for 10 days. The obvious advantages of these plant cell factory systems are ease of manipulation, speed, low cost, high protein yield, scalability and tight control of both upstream and downstream processing during manufacturing of these plant-derived biologicals.

Recently, plant-based systems for the expression of recombinant proteins for vaccines and therapeutics have led to the generation of products successfully assessed in clinical trials. IFN-α_{2b}), produced in transgenic duckweed [301], used to combat hepatitis C virus (HCV) was assessed in a Phase I clinical trial, while human glucocerebrosidase produced in transgenic carrot cell cultures to combat Gaucher's disease has progressed into a Phase III clinical trial [4]. Up till now, there are only few examples of recombinant proteins synthesized using transient expression systems entering clinical trials. A case study example is the personalized therapeutic vaccine for non-Hodgkin's lymphoma based on recombinant single-chain variable fragment (scFv) antibodies transiently produced in *Nicotiana benthamiana* plants by using a tobacco mosaic virus (TMV)-derived vector, assessed in a Phase I clinical trial [5]. Very recently, a H5N1 pandemic influenza vaccine based on the production of virus-like particles

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(VLPs) in *N. benthamiana* by using the hypertranslatable cowpea mosaic virus protein expression system (CPMV-HT) has also entered a Phase I [6].

It is a common notion that this way to produce biopharmaceuticals could soon replace the time-consuming procedures involved in bioproducts derived from stable transgenics, showing promise of high competitiveness towards 'classical' established methods.

While a number of excellent reviews have partly dealt with aspects of relevance to the field of transient expression in plants [1,7–16], in this article we tried to bridge the gaps, describing in thorough detail the two most promising transient expression approaches based on plant pathogen vectors, namely *Agrobacterium tumefaciens* strains and plant viruses with a wide range of expression and replication strategies.

Agroinfection

Agrobacterium-mediated transfer of genes from bacteria into plant cells in transient expression systems occurs via a specially constructed so-called binary vector family. These vectors consist of two parts: the first component is T-DNA (the segment delimited by the border sequences, the right [RB] and left [LB] border) and may contain multiple cloning sites, a selectable marker gene for transformed plant cells, a reporter gene and other genes of interest; and the second component is the vector backbone, which carries plasmid replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes for bacteria and optionally genes encoding plasmid mobilization functions [17,18]. The binary vectors have been used for many years not only for analyzing gene function but also for the production of biopharmaceuticals (Table 1). This approach uses the plant host *Lactuca sativa*, *Arabidopsis thaliana* or *Nicotiana tabacum*. Recently, *N. benthamiana* became the most widely used experimental host in plant virology, monoclonal antibodies (mAbs) and vaccine production [19].

An obvious advantage of transient *Agrobacterium*-based vaccine gene expression is speed. The full expression of a gene of interest in agroinjected leaves may be reached in 3–4 days after being infiltrated with *Agrobacteria* [20,21]. Another attractive feature of this system is simplicity. All experimental procedures do not require expensive supplies and equipment. Leaves of greenhouse-grown plants may be infiltrated by using a syringe without a needle, vacuum infiltration [22–25] or the 'wound-and-agrospray' inoculation method [26]. Supplementation of the infiltration media with either surfactants such as Triton X-100, Tween-20 or Silwet L-77 improved the levels of expression [20,25]. This method provides synchronous gene expression because *Agrobacterium* is known to simultaneously infect at least 96% of cells of injected leaves [27].

There are many factors influencing the yield and quality of transgenic proteins: promoter activity, gene silencing, codon usage, protein stability and subcellular targeting. Post-transcriptional

Table 1. Yield of recombinant molecules of immunological interest obtained by *Agrobacterium*-mediated nonviral transient expression systems[†].

Type of inoculation	Silencing suppressor	Pharmaceutical protein	Highest yield	Ref.
Nicotiana benthamiana leaf agroinfiltration	No	HIV-1 p24 (144 aa), p17/24 and Gag (500 aa)	From 44 µg/kg FW (Gag) up to 16,148 µg/kg FW (p24), partially purified	[118]
N. benthamiana leaf agroinfiltration	Yes	HIV-1 Nef (219 aa)	250 ng/g FW, affinity purified	[28]
N. benthamiana leaf agroinfiltration	No	HPV-16 L1 (531 aa) with VLP formation	400 µg/g FW	[119]
N. benthamiana leaf coagroinfiltration	Yes	SARS-CoV nucleocapsid protein (420 aa)	79 µg/g FW, partially purified	[29]
N. benthamiana leaf coagroinfiltration with TBSV p19	Yes	*Mycobacterium tuberculosis* ESAT6:Ag85B (342 aa)	100 µg/g FW, partially purified	[72]
Vacuum infiltration of lettuce (*Lactuca sativa* L.) with two populations of *Agrobacterium* encoding the mAb light and heavy chains	No	Full-size mAb (~160 kDa tetramer)	20–80 µg/g FW, affinity purified	[22]
Vacuum infiltration of *N. benthamiana* leaves with three populations of *Agrobacterium* encoding the mAb light, heavy chains and AMCV p19	Yes	Full-size mAb (~160 kDa tetramer)	50–100 µg/g FW, affinity purified	[106]
N. benthamiana leaf agroinfiltration with three populations of recombinant *Agrobacterium* encoding the mAb light, heavy chains and TBSV p19	Yes	Full-size mAb (~160 kDa tetramer)	100–300 µg/g FW, affinity purified	[202]

[†]The vector used in each of these cases was a 35S-based binary vector.
aa: Amino acid; AMCV: Artichoke mottled crinkle virus; FW: Fresh weight; HPV: Human papillomavirus; mAb: Monoclonal antibody; SARS-CoV: Severe acute respiratory syndrome coronavirus; TBSV: Tomato bushy stunt virus; VLP: Virus-like particle.

gene silencing (PTGS) is one of the reasons why leaves infiltrated by *Agrobacteria* usually express low amounts of protein, but coinjection with genes encoding silencing suppressors increases protein accumulation [28–30]. Moreover, correct protein folding and protein stability in the target cell compartment have great influence on vaccine protein yield. Recently, different strategies have been adopted to improve protein accumulation:

- Green fluorescent protein (GFP) as a carrier and fusion partner helped antigen folding and increased hepatitis B virus (HBV) surface antigen (HBsAg) [31] and chicken anemia virus (CAV) VP1, VP2 and VP3 [32] production;
- Hydrophobin sequence from *Trichoderma reesei* increased the expression levels of plant recombinant proteins transiently expressed in *N. benthamiana* plants by *Agrobacterium* infiltration [33];
- The construct harboring the complete Newcastle disease virus (NDV) hemagglutinin–neuraminidase (HN) glycoprotein gene with its own signal peptide, fused to a KDEL retention peptide, increased accumulation of HN [34];
- β-glucuronidase (GUS) used as a stable fusion partner enhanced the accumulation of a peptide derived from canine parvovirus within the cytoplasmic environment [35];
- Antibody Fc fragment was used to increase protein stability and yield of HIV-1 p24 [36].

Leaf agroinfiltration is a widely exploited approach, but also the plant root is considered an alternative system for foreign protein production. The gene encoding a vaccine protein may be transformed into root cells via direct virus inoculation [37,38] or *Agrobacterium*-mediated delivery of TMV vectors in *N. benthamiana* clonal root cultures, using either *A. tumefaciens* [30] or *Agrobacterium rhizogenes* [39]. This system has several advantages in that it represents a continuous organ culture system for contained manufacturing, easy to scale-up with higher expression levels if compared with leaf infiltration.

Plant virus-based vectors

Since 1984, when Ahlquist *et al.* first achieved *in vitro* synthesis of infectious brome mosaic virus (BMV), RNAs from full-length cDNA copies of postive-strand RNA viruses of both plants and animals have been produced successfully *in vitro* and *in vivo* [40]. The synthesis of infectious transcripts *in vitro* was a hallmark for plant virus-based vectors for devising and expressing recombinant proteins in field-grown plants [10]. The gene of interest is delivered to plant cells using infectious nucleic acid copies of the vector or, preferentially, as mature viral particles. The plant virus-based vector technology has become a rapidly growing research area with significant applications in biopharmaceutical production (Table 2).

Tobamoviruses

Complete sequencing of the TMV genome and the synthesis of the T7 RNA-polymerase-directed full-length infectious transcripts *in vitro* opened the way for construction of 'added-gene' plant virus-based vectors. TMV U1 strain RNA encodes four major proteins. The 126- and 183-kDa replicase proteins are translated from the first open reading frame (ORF) within the genomic RNA, the latter by occasional read-through of the amber stop codon for the 126-kDa protein. The 30-kDa movement protein (MP) and 17.4-kDa coat protein (CP) are expressed via individual 3′-coterminal subgenomic (sg) RNAs from the 3′-proximal ORFs [41]. Whereas the MP and the CP are dispensable, the 126- and the 183-kDa replicase proteins are required for viral RNA replication. The sg bicistronic intermediate-length RNA-2, called I_2 sgRNA, is translated to produce the MP, whereas the 3′-proximal CP gene of I_2 RNA is translationally silent [42]. This gene is expressed from the small monocistronic sgRNA called low-molecular component (LMC). The vectors based on TMV [43], crucifer-infecting tobamovirus (crTMV) turnip vein clearing virus (TVCV) [44] and tomato mosaic virus (ToMV) [45] genomes exploit the sg promoter of the CP gene, providing the synthesis of the protein of interest directly from its own or an additional CP sg promoter (Figure 1). In RNA plant viruses, the CP sgRNA promoter extends downstream of the transcription initiation site and its activity is the highest in vectors where the CP is fused with a small immunogenic epitope [14]. The internal ribosome entry site (IRES) sequence presents an alternative way of vector-directed foreign gene expression. Translation of TMV U1 strain CP mRNA occurs by traditional cap-dependent ribosome scanning. However, vectors based on the genome of crTMV present another expression strategy where the IRES can be exploited. IRES-mediated translation is intrinsically less efficient than cap-dependent translation but can provide expression of polycistronic templates [46].

Potexviruses

Potexviruses have monopartite, positive-strand RNA genomes encoding five ORFs. The 5′ end has a cap and the 3′ end has a polyA tail. The first ORF encodes the viral replicase. The central region of the genome encodes three overlapping ORFs, known as the triple gene block (TGB). These proteins are required for virus cell-to-cell movement [47,48]. The final ORF is the viral CP, which is required for virion assembly and virus cell-to-cell movement. The first potato virus X (PVX)-based vector was designed according to the 'added gene' strategy, where reporter gene (GFP) was cloned under control of duplicated CP sg promoter resulting in pPVX201 where viral RNA synthesis was 35S cauliflower mosaic virus (CaMV) promoter dependent (Figure 1) [49]. The majority of PVX-based vectors for different protein of interest production utilize pPVX-201 as a template [26,50]. But the stability of the added gene PVX-based vector is discussed and is likely dependent on the insert length [51]. Therefore, several 'replaced gene' variants were also designed. Giritch *et al.* made a so-called 'deconstructed' viral vector (see section 'The MagnICON expression system') in which the CP gene was substituted with GFP or antibody light-/heavy-chain genes (Figure 1) [52]. Removal of CP led to a higher level of target protein expression. Another group constructed a PVXdt–GFP viral vector by deleting the TGB and CP gene. GFP expression was directed by the 25-K sg promoter (Figure 1) [53]. This variant was completely movement dysfunctional but gave considerable increase

Table 2. Yield of recombinant molecules of immunological interest obtained by plant virus-mediated transient expression systems.

Virus vector	Type of inoculation	Pharmaceutical protein	VLP	Highest yield	Ref.
TMV	TMV-30B-based vector-directed RNA transcript inoculation of spinach leaves	HIV-1 Tat protein (86 aa)	No	0.3–0.5 mg/g FW	[120]
TMV	TMV-30B-based vector-directed RNA transcript inoculation of *Nicotiana benthamiana* leaves	HPV-16 L1 protein (531 aa)	Yes	~0.03 x 10^{-3} mg/g FW	[121]
TMV	ToMV-TocJ-based vector directed RNA transcript inoculation of *N. benthamiana* leaves	The dengue virus envelope protein (102 aa fragment)	No	~0.1 mg/g FW	[122]
TMV	*N. benthamiana* leaf agroinfiltration	*Mycobacterium tuberculosis* Ag85B and ESAT6 proteins (342 aa)	No	0.8–1.0 mg/g FW	[72]
TMV	TMV TTO1A vector-directed RNA transcript inoculation of *N. benthamiana* leaves	Human scFv proteins (~30 kDa) derived from human tumor immunoglobulin genes of non-Hodgkin's lymphoma patients	No	100–800 µg/ml of leaf interstitial fluid	[5]
TMV	TMV launch vector pBID4 agroinfiltration of *N. benthamiana* leaves	hGH (~30 kDa)	No	0.7 mg/g FW	[123]
TMV and PVX	*N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anticancer full-size mAb (~160 kDa)	No	100–300 µg/g FW, affinity purified	[202]
TMV and PVX	MagnICON: *N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anticancer full-size mAb (~160 kDa)	No	0.3–0.5 mg/g FW, affinity purified	[52]
TMV and PVX	MagnICON: *N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anti-West Nile virus mAb (~160 kDa)	No	0.8 mg/g FW leaves, affinity purified	[108]
TMV	MagnICON: *N. benthamiana* leaf agroinfiltration	*Yersinia pestis* F1 (362 aa) and V (150 aa) antigens	No	0.3–0.5 mg/g FW; 1.2 mg /g FW (purified product)	[76]
TMV	MagnICON: *N. benthamiana* leaf agroinfiltration	VV B5 (275 aa) antigenic domain	No	0.1 mg/g FW	[124]
TMV	MagnICON: *N. benthamiana* leaf agroinjection	*Plasmodium* antigen PyMSP4/5 (33 kDa)	No	1–2 mg/g FW	[125]
TMV	MagnICON: *N. benthamiana* leaf agroinfiltration	NV CP (~58 kDa)	Yes	0.8 mg/g FW	[82]
PVX	Inoculation of *N. benthamiana* leaves with pPVX201 vector-based cDNA construct	HBV nucleocapsid protein (21 kDa)	Yes	0.005–0.01 mg/g FW	[50]
PVX	Agroinfiltration of *N. benthamiana* leaves with pPVX201-based vector cDNA construct	*M. tuberculosis* ESAT6 antigen fusion with PVX CP (31 kDa)	No	0.5–1% of the TSP	[126]
PVX and TMV	*N. benthamiana* leaf agroinfiltration	GFP fused with CAV VP1, VP2 and VP3 (40–51 kDa)	No	1.2–5.4% of the TSP	[32]
BeYDV	*N. benthamiana* leaf agroinfiltration	HBc (21 kDa) and NV CP (58 kDa)	Yes	HBc: 0.80 mg/g FW; NV CP: 0.34 mg/g FW	[71]

aa: Amino acid; BeYDV: Bean yellow dwarf virus; BHV: Bovine herpesvirus; CAV: Chicken anemia virus; CP: Coat protein; CPMV: Cowpea mosaic virus; FMDV: Foot-and-mouth disease virus; FW: Fresh weight; GFP: Green fluorescent protien; HBc: Hepatitis B virus core antigen; hGH: Human growth hormone; mAb: Monoclonal anitbody; NV: Norwalk virus; PPV: Plum pox virus; PVX: Potato virus X; RHDV: Rabbit hemorrhagic disease virus; scFv: Single-chain variable fragment; TMV: Tobacco mosaic virus; ToMV: Tomato mosaic virus; TSP: Total soluble protein; VLP: Virus-like particle; VV: Vaccinia virus.

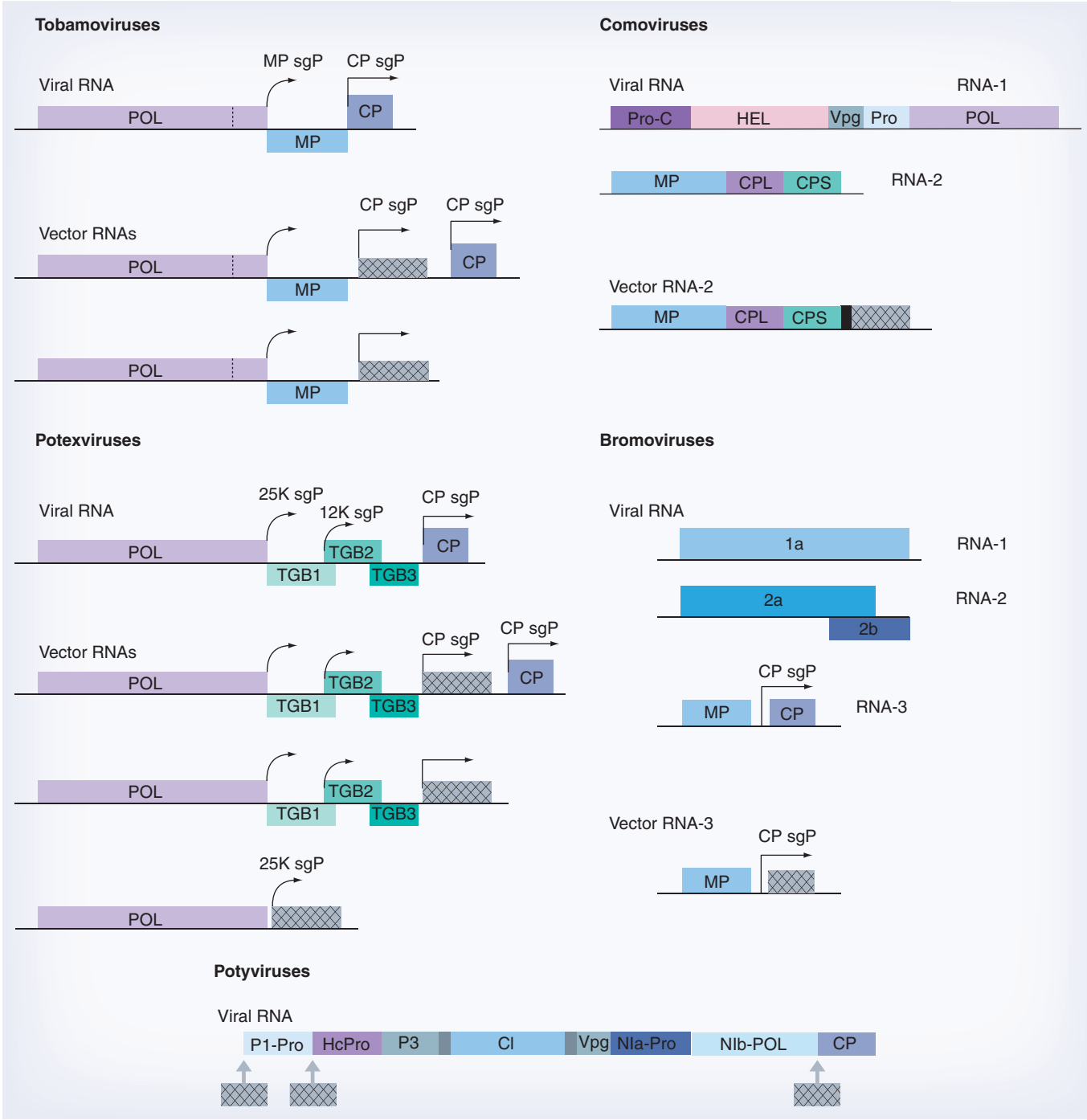

Figure 1. Schematic representation of viral genomes and vectors used to express heterologous peptides and proteins in plants. Target protein genes are shown hatched and their positions in the viral vectors are indicated. 2A catalytic peptide is marked with a black box. Dashed line represents a leaky termination codon.
1a, 1b and 2b: Components of replicase; CI: Cylindrical inclusion protein; CP: Coat protein; CPL: Large coat protein; CPS: Small coat protein; FMDV: Foot-and-mouth disease virus; HC-Pro: Helper component proteinase; Hel: Helicase; MP: Movement protein; NIa-Pro: Viral proteinase; NIb-POL: RNA-dependent RNA polymerase; P3: Protein P3; POL: RNA polymerase; Pro: Proteinase; ProC: Proteinase cofactor; sgP: Subgenomic promoter (marked with arrows); TGB1–3: Components of the triple gene block; VPg: Virus protein genome linked.

in target protein production achieved when delivered into plant cells by agroinjection (together with the silencing suppressor P19).

The other strategy in PVX-based vector design is the fusion of target protein to CP. There are two variants: target protein fused to CP N-terminus [54] or linked to the CP via the foot-and-mouth disease virus (FMDV) 2A catalytic peptide [55]. The 2A

sequence promotes cotranslational cleavage of the fusion protein and maintains virus infectivity allowing the synthesis of both cleaved and recombinant CPs [55]. These approaches are usually used for peptide display.

Potyviruses

Potyviral-based vectors exploit different strategies for the expression of foreign proteins. Their genome is a single-stranded, positive-sense RNA translated into a single polyprotein that is further processed by virus-encoded proteases. The first potyvirus tagged with a foreign gene was tobacco etch virus (TEV) engineered to express the GUS reporter enzyme fused to the N-terminus of helper component proteinase (HC-pro) [56]. A plum pox potyvirus (PPV)-based vector has been constructed for the expression of full-length vaccine proteins. The foreign sequences were cloned between the NIb replicase and CP cistrons (Figure 1). The heterologous protein is split from the rest of the potyviral polyprotein by cleavage at the site where the NIb and CP proteins are originally separated and at an additional NIa protease recognition site engineered at the N-terminus [57]. Several other members of the group are used in vector design and foreign protein production. The next generation of potyvirus-based vectors allows simultaneous expression of two [58] or more [59] target proteins from one vector (Figure 1).

Bromoviruses

Cucumber mosaic virus (CMV) and alfalfa mosaic virus (AMV) are the members of this group. They have segmented, tripartite linear ssRNA-positive genome composed of RNA1, RNA2 and RNA3. Each genomic segment has a 3′-tRNA-like structure and a 5′-cap. RNA1 and RNA2 encode proteins 1a and 2a, respectively, both involved in genome replication and internal transcription of sgRNA4 from the minus-strand copy of RNA3. RNA3 and sgRNA4 are translated into movement and capsid proteins, respectively. In the first CMV-based vector, CP gene was usually replaced by foreign gene (Figure 1) that prevented the CP-dependent cell-to-cell movement. In this case, CP was provided *in trans* to ensure proper cell-to-cell movement [60], otherwise the viral vector is limited to the initially infected cells. In a more advanced version, CMV inducible viral amplicon (CMViva), one of the key components of the viral replicase (from RNA1), was under the control of a tightly regulated expression system inducible by estradiol. All three CMV components were engineered on a single Ti plasmid. As compared with analogous vectors under control of the 35S promoter, the overall yield of target protein (human α1-antitrypsin [AAT]) obtained with CMViva was several-fold higher [61]. The next step in development of a CMV-based system was to retrieve cell-to-cell movement. Previous studies have shown that deletion of the C-terminal 33 amino acids of CMV 3a MP enabled viral cell-to-cell movement independently of CP, although at lower efficiency [62]. A new CMV-based expression vector was designed that exploits the mutant 3a MP for CP-independent cell-to-cell movement [63]. Two (RNA1 and RNA2) or all three CMV components were engineered on a single Ti plasmid and CMV genome sequences were placed under the control of the 35S CaMV promoter. High yields of GFP (~450 mg/kg leaf tissue) and human growth hormone (hGH; ~170 mg/kg leaf tissue) were achieved in *N. benthamiana*. CMV-based expression vectors are particularly attractive because the virus has a very broad host range, including more than 1000 monocot and dicot plant species.

Another broad host range virus is AMV. Several species of legumes were screened for transient protein expression using AMV-based expression system [64]. This system consists of three constructs expressed under the control of CaMV 35S promoter: one for RNA1 and RNA2 (encoding AMV viral replicase), a second encoding the viral CP required for genome activation and replication and a further construct for RNA3 in which the CP coding region is replaced by the foreign gene. Expression levels of 420 ± 26.24 mg GFP/kg fresh weight (FW) in the green pea variety speckled pea were achieved. High expression levels were also achieved for the anthrax protective antigen fused to lichenase (LicKM–PAD4) [64].

Comoviruses

Cowpea mosaic virus, the type member of the comoviruses group, has a narrow host range, normally infecting legumes other than *N. benthamiana* as experimental host. The genome of CPMV consists of two separately encapsidated positive-strand RNA molecules: RNA1 and RNA2. Each genomic RNA has a viral protein genome (VPg) covalently linked to the 5′ end, a 3′-polyA tail and encodes a single ORF whose expression is achieved through the subsequent processing of a precursor polyprotein. RNA1 encodes proteins involved in the replication of viral RNAs and polyprotein processing. RNA2 encodes the MP and the two CPs, which are essential for cell-to-cell movement and systemic spread. The proteinase responsible for processing both the RNA1 and RNA2 polyproteins is encoded by the RNA1 [65]. When designing CPMV-based vectors, usually RNA1 component remains unmodified as it contains genes essential for replication. As for the RNA2, two different approaches are used. The first implies foreign gene insertion in RNA2 resulting in an increase in size which is still functional for systemic spreading. The second, most efficient, involves insertion of the foreign gene as an in-frame C-terminal fusion to the RNA2-encoded polyprotein (Figure 1). The rescue of target protein is performed by an inducible proteolytic shunt through the 2A catalytic peptide from FMDV inserted upstream of the foreign protein sequence [66]. The first strategy is usually pursued when production of viral (or virus-like) particles is devised, while the second allows larger inserts to be incorporated, but abolishes the ability of the virus to spread both within and between plants. The system relies on the observation that the sequences necessary for replication of RNA2 by the RNA1-encoded replicase lie exclusively at the 5′ and 3′ ends of the RNA. This allows most of the RNA2 ORF to be deleted without affecting the ability of RNA2 to be replicated. Unfortunately, such vector system lacks natural CPMV silencing suppressor [67] so another component (i.e., Hc-Pro from potato virus Y) should be supplied. This is usually done through agroinfiltration that allows simultaneous delivery of several genetic expression constructs in plant cells. Utilization of 'deleted' variant of RNA2 gives higher yield of target protein and is clearly advantageous in

case of antibody production [68]. Moreover, binary vectors simplifying cloning and expression steps with the CPMV system were designed. CPMV expression cassettes and the sequence encoding P19 have been incorporated into the T-DNA region of the resulting vector allowing high-level expression of multiple polypeptides by infiltration of a single construct [65].

Geminiviruses

Viruses of this family possess single-stranded circular DNA genomes that replicate to very high copy number in the nuclei of infected plant cells. Owing to these unique features, the potential for using the DNA-containing geminiviruses as extrachromosomal gene amplification vectors for the expression of useful proteins in plants has been recognized [69]. At present, the improved expression of recombinant GFP using a replicating vector based on beet curly top virus in leaf disks and agroinfiltrated *N. benthamiana* leaves was observed [70]. In addition, a vector replicon based on the bean yellow dwarf virus (BeYDV) DNA genome was created recently [71]. A system composed of two vectors containing different portions of the BeYDV genome was constructed. The 'Rep supplying vector' encodes Rep, whose expression can be controlled by specific promoters and the LSL vector that contains the gene of interest and the *cis*-acting elements (the long and short intergenic regions, LIR and SIR, respectively) required for replication of the viral genome. It was shown that formation of BeYDV-based replicons in bombarded tobacco NT-1 cells resulted in increased transient expression of the GUS protein. The delivery of added vector components increased protein production [71]. Codelivery of the BeYDV-derived vector and the Rep/RepA-supplying vector by agroinfiltration of *N. benthamiana* leaves resulted in efficient replicon amplification and protein production within 5 days. Coexpression of the tomato bushy stunt virus (TBSV) P19 protein enhanced the stability of the mRNA and increased protein yield. A single replicon vector containing a built-in Rep/RepA cassette drove protein expression to levels similar to the three-component system, even in the absence of P19 silencing suppressor.

The MagnICON expression system

The *Agrobacterium*-delivered plant viral vectors in the transient expression systems exploits the RNA polymerase (Pol) II–mediated nuclear export route including 5′-end capping, splicing and 3′-end formation. In contrast to DNA geminiviruses, plant RNA viruses replicate in the cytoplasm and are not adapted to nuclear splicing machinery, which recognizes and removes cryptic introns from viral RNA leading to degradation. The *Agrobacterium*-delivered, so-called 'first-generation' TMV and PVX vectors, have low production capacity with protein yields quite low (0.04–0.3% of the total soluble protein [TSP]), requiring coinjection of plasmids encoding silencing suppressors such as tombusvirus p19, potyvirus P1/HC-Pro [26,32,72], inhibitors of pectin methylesterase [73] or Pol II-directed short noncoding RNAs [201].

To adjust TMV infectious copy transcription to *Agrobacterium*-mediated vector delivery, Gleba and coworkers developed a new generation expression platform, also known as 'Magnifection', a new approach for expressing recombinant biopharmaceuticals in plants [8,74]. The MagnICON system includes advantages of *Agrobacterium*-mediated delivery and upgraded TMV-based vectors where putative cryptic splice sites were removed and multiple plant introns were inserted [27,44].

The idea of 'deconstructed virus' (pro-vector) is based on the efficient assembly of DNA modules obtained by recombination in plant cells. In this system, *Agrobacterium*-delivered plasmids encoding 5′-part and 3′-part of a viral vector and, as an additional component, the plasmid encoding an integrase, essential for full vector assembly *in planta* are used. To make assembly of the viral vector precise and prevent mutations that could occur during recombination, a fragment of plant intron was inserted into each part of provector: the donor site just upstream of recombination site in the 5′-part and the acceptor site downstream of recombination site in the 3′-part. Thus, the recombination site with possible mutations is deleted during splicing [44].

The adopted strategy of deconstructing and reconstructing the viral RNA vector provides enhanced versatility and efficiency due to varying gene combinations, targeting signals and tags can be tested without the need to engineer each individual variant construct [44]. The cloning procedure is made even simpler with 'golden-gate' shuffling method allowing to assemble (in one step and one tube) at least nine separate DNA fragments into an acceptor vector, obtaining different combinations and then choosing the best variant for each protein of interest [75]. This system proved to be very efficient for the production of different proteins of interest and is now widely used [52,76].

Antigens or antigen domain production

There are two main methods for the production of foreign proteins in plant transient expression systems: antigens may be expressed in the plant cell as a single vaccine protein or in fusion with a protein carrier (Table 3); or antigenic peptides may be localized (exposed) on the surface of plant virions (Table 4).

Recombinant soluble antigens

Transient expression strategies can be used to express in plants both soluble or self-assembling antigens. As for soluble whole antigens or antigen domains, polypeptides derived from several different pathogens have been produced mainly in *Nicotiana* species with varying recoveries depending on the expression strategy adopted but generally with expression levels significantly higher compared with those obtained through nuclear transformation. The first original paper in this area described the expression of the structural VP1 protein of FMDV using a TMV-based gene insertion vector with a yield of approximately 0.5–1 µg/g FW [77]. Since then transient expression underwent a fundamental evolution thanks to the development of modular deconstructed expression systems and also to the exploitation of silencing suppressors, codon optimization, expression targeting to specific subcellular compartments and even identification of optimal harvesting time [10]. With these devices, it is now possible to obtain up to 800 µg of the protein of interest per gram of fresh leaf tissues [72]. At present, the major bottleneck of the technology

Table 3. Immunological efficacy of respresentative antigens or antigen domains transiently expressed in plant tissues.

Antigen (pathogen/disease)	Plant	Expression level	Form of delivery	Adjuvant†	Dose (animal)	Doses (n) and delivery route	Type of response	*In vitro* neutralization assay or challenge	Ref.
VP1 (FMDV)	*Nicotiana benthamiana*	50–150 µg/g FLW	FE	+	0.5–1 µg/150 µl (M)	Five ip.	Humoral	+	[77]
R9 mimotope (HCV)–cholera toxin B	*N. benthamiana*	6–80 µg/g FLW 0.2% TSP	FE	+	0.5–1 µg/30 µl (M)	Five in.	Humoral	ND	[127]
VP60 (RHDV)	*Nicotiana clevelandii*	ND	FE	+	1 ml (R)	Two sc.	Humoral	+	[128]
E7 (HPV-16)	*N. benthamiana*	3–4 µg/g FLW	FE	-	0.5 µg/500 µl (M)	Four sc.	Humoral Cellular	+	[129]
gDc (BHV-1)	*N. benthamiana*	15–20 µg/g FLW	FE	+	200 µg (M) 5 g (C)	One ip. (M) Seven (half dose im. and half dose sc.) (C)	Humoral Cellular	+ (C)	[130]
GA733-2 (colorectal cancer)	*N. benthamiana*	ND	PA	+	20 µg (M)	One sc. + two ip.	Humoral Cellular	ND	[131]
SAG1 (*Toxoplasma gondii*)	*Nicotiana tabacum*	6–10 µg/g FLW 0.06–0.1% TSP	FE	+	200 ng (M)	Four sc.	Humoral	+	[132]
Tat (HIV-1)	*Spinacia oleraceae*	300 µg/g FLW	FL	-	300 µg (M)	Three oral	Humoral	ND	[120]
F1 and V (*Yersinia pestis*)	*N. benthamiana*	1–2 mg/g FLW	PA	+	25 µg (GP)	Three sc.	Humoral	+	[76]
PA (*Bacillus anthracis*)–lichenase	*N. benthamiana*	ND	PA	+	100 µg (M)	Three ip.	Humoral	+	[133]
B5 (VV)	*N. benthamiana*	100 µg/g LL	PA	+	2 µg (M)	Two sc. + one ip.	Humoral	+	[124]
E7 (HPV-16)–lichenase	*N. benthamiana*	400 µg/g FLW	PA	+	40 µg (M)	Five sc.	Humoral Cellular	+	[134]
F1 and V (*Yersinia pestis*)–lichenase	*N. benthamiana*	120–380 µg/g FLW	PA	+	25–250 µg (MC)	Three sc.	Humoral	+	[135]
Domain III $Env_{298–400}$ (DENV2)	*N. benthamiana*	5.6 µg/g FLW 0.28% TSP	PA	+	10–20 µg (M)	Nine (four × 10 µg and five × 20 µg) im.	Humoral	+	[122]
$HA_{17–532}$ (influenza A virus, H5N1)	*N. benthamiana*	60 µg/g FLW	PA	+	15–45 µg (M) 45–90 µg (F)	Three3 sc.	Humoral	+ (F)	[136]

†Antigen delivered with (+) or without (-) adjuvant.
BHV: Bovine herpes virus; C: Cattle; CAV: Chicken anemia virus; DENV: Dengue virus; DLP: Dried leaf powder; F1: Fraction 1; F: Ferret; FE: Foliar extract; FL: Fresh leaves; FLW: Fresh leaves weight; FMDV: Foot-and-mouth disease virus; GP: Guinea pig; HA: Hemagglutinin; HCV: Hepatitis C virus; HPV: Human papilloma virus; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; LL: Lyophilized leaves; M: Mouse; MC: Macaque; ND: No data; PA: Purified antigen; R: Rabbit; RHDV: Rabbit hemorrhagic disease virus; SARS-CoV: Severe acute respiratory syndrome-coronavirus; sc.: Subcutanous; TSP: Total soluble protein; V: V antigen; VV: Vaccinia virus.

Table 3. Immunological efficacy of respresentative antigens or antigen domains transiently expressed in plant tissues (cont.).

Antigen (pathogen/disease)	Plant	Expression level	Form of delivery	Adjuvant†	Dose (animal)	Doses (n) and delivery route	Type of response	*In vitro* neutralization assay or challenge	Ref.
HA_{17-532} (influenza A virus, H5N1)	*N. benthamiana*	ND	PA	+	2.5–5 µg (M)	Three sc.	Humoral	+	[137]
HA_{1-330} (influenza A virus, H5N1)	*N. benthamiana*	1 µg/g FLW	PA	+	10 µg (M)	Two im.	Humoral	-	[138]
PyMSP4/5 *(Plasmodium yoelii)*	*N. benthamiana*	1–2 mg/g FLW 10% TSP	DLP	+	100 µg (M)	Five oral (after one DNA vaccine dose)	Humoral	ND	[125]
N (SARS-CoV)	*N. benthamiana*	79 µg/g FLW 0.8–1% TSP	FE	+	2–4 µg (M)	Four ip.	Humoral	ND	[29]

†Antigen delivered with (+) or without (-) adjuvant.
BHV: Bovine herpes virus; C: Cattle; CAV: Chicken anemia virus; DENV: Dengue virus; DLP: Dried leaf powder; F1: Fraction 1; F: Ferret; FE: Foliar extract; FL: Fresh leaves; FLW: Fresh leaves weight; FMDV: Foot-and-mouth disease virus; GP: Guinea pig; HA: Hemagglutinin; HCV: Hepatitis C virus; HPV: Human papilloma virus; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; LL: Lyophilized leaves; M: Mouse; MC: Macaque; ND: No data; PA: Purified antigen; R: Rabbit; RHDV: Rabbit hemorrhagic disease virus; SARS-CoV: Severe acute respiratory syndrome-coronavirus; sc.: Subcutanous; TSP: Total soluble protein; V: V antigen; VV: Vaccinia virus.

seems to be represented by the extraction and purification steps. Even if *in vivo* experiments often demonstrated the ability of plant-derived antigens to protect animals from challenge, the immunological properties of plant-derived recombinant antigens or antigen domains have been mostly tested by orally delivering to mice (through gavage) powdered freeze-dried leaves or plant extracts. Alternatively, mice have been injected with plant extracts (Table 3). This, of course, prevents a correct evaluation of the immune response that can be positively or negatively affected by unidentified plant components. Several strategies have been evaluated to facilitate purification of the recombinant antigen other than classical affinity tags [36,78,79].

Plant-derived virus-like particles

An ideal vaccine against viral pathogens should mimic the native virus to induce a protective immune response. These vaccines traditionally based on live-attenuated or inactivated viruses are effective in inducing both cellular and humoral immune responses. However, a major concern about the live-attenuated vaccines is their potential ability to revert to a pathogenic form [80]. In this respect, a novel class of VLP-based vaccines provides a promising way to overcome safety concerns. VLPs are macromolecular structures that morphologically resemble those of the virus from which they are derived and consist of one or several self-assembling viral proteins expressed through recombinant technologies. For both animal and plant viruses, there are several examples described in the literature in which the structural proteins retain their ability to self-assemble in the absence of the viral genome. These features make VLPs safe and effective vaccine tools for inducing strong B- and T-cell-mediated immune responses. Moreover, VLPs could be used not only as vaccine against the cognate virus but also as carriers for an efficient presentation of foreign epitopes to the immune system.

Pioneering work on hepatitis B core antigen (HBcAg)-derived VLPs was carried out using the MagnICON transient expression system in *N. benthamiana* leaves. High levels of antigen were obtained (2.38 mg/g FW) and immunogenicity assayed in mice [81]. The results clearly showed that plant-derived HBcAg VLPs were able to induce specific serum IgG and intestinal IgA when administered mucosally in mice without adjuvants. The same system was successfully used for the production Norwalk virus (NV) VLPs [82]. Similarly to HBcAg, NV VLPs were produced at high levels (0.86 mg/g FW) and elicited both systemic and mucosal immune responses.

Rapid and cost-effective vaccine production is essential to allow rapid response to newly emerging viruses. Influenza virus-derived VLPs in agroinfiltrated *N. benthamiana* leaves could therefore represent a novel vaccine technology against possible influenza pandemics. In fact, the results obtained by D'Aoust and colleagues showed that two doses of plant-produced influenza H5-VLPs were able to protect mice not only from lethal homologous virus challenge but also against heterologous strains [6,83].

An attractive approach for the production of epitope-based plant-derived vaccine formulations is the construction of chimeric VLPs based on plant viruses displaying immunogenic peptides. Several plant viruses (e.g., CPMV, papaya mosaic virus and PVX)