REVIEW

INFECTIOUS FULL-LENGTH CLONES OF PLANT VIRUSES AND THEIR USE FOR CONSTRUCTION OF VIRAL VECTORS

A. NAGYOVÁ[1], Z. ŠUBR[2*]

[1]Faculty of Natural Sciences, Commenius University, Mlynská dolina, 842 15 Bratislava, Slovak Republic; [2]Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

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Summary. – In the last two decades, preparation of infectious RNA or DNA clones of plant viruses has transformed to a standard laboratory technique, providing an excellent tool for the research of viral gene functions and virus-host interactions. A full-length clone can be transformed *in vitro* to a viral vector expressing foreign poly/oligopeptides in soluble form or fused to a viral capsid protein**.** The use of the plants as producers of antigens, allergens, and other pharmaceuticals is cheaper than the use other eukaryotic bioreactors. Transient expression of proteins using the vector technique has several advantages in comparison with a transgenic approach. Virus-induced gene silencing vectors have been widely adapted for research of plant genes functions and screening of plant genomes for resistance genes. The potential instability of the constructs remains the main problem of virus vector-based techniques leading to a wild-type virus recovery by recombination. A novel approach including "a deconstructed virus-strategy" may overcome these difficulties. A preparative-scale production of numerous biologically active compounds in the various plants using viral vectors are expected in the near future.

Key words: infectious cDNA; infectious transcripts; plant viral vectors; transient expression; vaccines

Contents:

1. Introduction
2. Use of restricted genome parts of plant viruses
3. Use of complete genomes of plant viruses
3.1. Infectious clones of plant viruses
3.1.1. Infectious transcripts
3.1.2. Infectious cDNA
3.1.3. Transfection of plants
3.1.4. Infectivity of prepared clones
3.1.5. Influence of non-viral sequences
3.2. Construction of viral vectors
3.2.1. Gene exchange
3.2.2. Gene insertion
3.2.3. Epitope presentation (CP fusion)
3.2.4. Complementation systems
3.3. Application in research of viral and plant genomes
3.4. Large-scale production of recombinant proteins in plants
3.5. Prospective strategies of viral vectors construction
4. Conclusions

*Corresponding author. E-mail: virusubr@savba.sk; fax: +4212-54774284.

Abbreviations: AMV = Alfalfa mosaic virus; CaMV = Cauliflower mosaic virus; CFP = cyan fluorescent protein; CMV = Cucumber mosaic virus; CP = capsid protein; CPMV = Cowpea mosaic virus; GFP = green fluorescent protein; PLRV = Potato leafroll virus; PVA = Potato virus A; PVX = Potato virus X; TMV = Tobacco mosaic virus; VIGS = virus-induced gene silencing; VpG = viral genome-linked protein; YFP = yellow fluorescent protein

1. Introduction

The viruses of plants served as important model systems in the history of biology. In fact, many key steps in the research leading to the modern knowledge of natural eukaryotic systems and to the current level of biotechnology were performed on the plant virus models. Viruses are intracellular parasites exploiting the host biosynthetic apparatus for their replication and code for a relatively restricted amount of own genetic information. The plant viruses belong to the smallest known viruses and the ability of some viral genes to redirect a host cell activity in favor of their replication is especially notable. The respective viral taxons differ by the type of the genome (DNA/RNA, single/double-stranded, sense/antisense/ambisense, linear/circular, monopartite/segmented) containing different terminal structures that relates to their replication strategy and gene implementation, what is important in the competition for energy and material sources, for transcriptional and translational apparatus of the host plant as well as for braking of its natural defense mechanisms (Hull, 2002).

Therefore, the plant viruses have to be considered as a potential source of genes and regulation elements usable for the construction of transgenic plants with specific features, or for transient heterologous expression of different polypeptides. These applications may be divided in two groups: 1/ the use of particular viral sequences, and 2/ the use of complete (although modified) viral genomes.

2. Use of restricted genome parts of plant viruses

As a regulation element, the 35S promoter of Cauliflower mosaic virus (CaMV) found the broadest application. It is a relatively strong constitutive promoter without marked tissue specifity (Haas *et al.*, 2002; Guilley *et al.*, 1982). The promoter is weak for some purposes and for that reason, serial arrangement of several copies is often used (Kay *et al.*, 1987). The promoters of viruses may have different host or tissue specificity. For example, the promoters of the Commelina yellow mottle virus or Tomato yellow leaf curl virus – China may be used for the directed expression in the vascular tissues of mono- and dicotyledonous plants (Guan and Zhou, 2006; Medberry *et al.*, 1992). In some cases, it may be useful to coordinate the expression of two or more genes in plants by several different appropriate promoters. Subgenomic promoters are localized in the middle parts of viral genomes. A different (higher) strength of the subgenomic promoter may serve as a regulatory mechanism affecting the quantity of expressed proteins (Ahlquist and French, 1997).

Translational enhancers are important for the competition of the virus with host transcripts. The Ω enhancer from the non-coding region of Tobacco mosaic virus (TMV) is the best known and the most important one. The Ω sequence increases the translation efficiency in transgenic plants as much as ten times (Schmitz *et al.*, 1996; Gallie and Kado, 1989).

Until now, particular proteins of plant viruses have not been commercially used, although some of them are promising for molecular biology applications, e.g. polymerases, endonucleases, and proteases. However, the specific viral genes are often inserted artificially in the plant genomes in order to prepare virus-resistant transgenic plants (Goldbach *et al.*, 2003).

3. Use of complete genomes of plant viruses

A modified viral genome may be used for the introduction of the foreign genes into plants. Essentially, the viral vector is an infectious clone of the virus with an introduced suitable cloning site and with the non-functional gene for natural virus transmission. A transient gene expression is an alternative to material- and time-consuming preparation of transgenic plants. The main problem of the viral vector is the potential instability, especially when the genome is significantly prolonged in comparison with the genome of the wild virus (Hammond, 2005). In this case, the recombination may lead to a reversion of the genome to the original size preferred in the process of nucleic acid coating and particle assembly.

On the other hand, the viral vectors have some advantages. The expression of viral vectors is simply controllable and the potential risk of unwanted horizontal gene·dransfer is minimized, as well as the vertical transfer to the progeny. The viral vector replicates independently of the host genome like its parental virus. From the practical point of view, viruses multiplying to a high titer in the plants are most suitable for the vector construction. Massive expression of viral (and virus-associated) genes occurs usually in 1–2 weeks after inoculation and the yield of expressed product may reach as much as 2 g/kg of the green mass (Walmsley and Arntzen, 2000). Simple purification from the infected plants is also advantageous. Mechanical transmissibility of the parental virus is not necessary, but it is preferred. An essential requirement is the availability of the viral genome in the form of a DNA clone.

3.1. Infectious clones of plant viruses

The first attempts to prepare viral vectors were performed with the DNA viruses. The DNA isolated from the vector may be considered as infectious clone. For example, high amount of vectors based on the genomic DNA of geminiviruses accumulate in the host cells. However, they have a limited cloning capacity and the reconstitution of the

wild-type virus often occurs (Hefferon and Dugdale, 2003; Scholthof *et al.*, 1996).

The discovery and commercial availability of enzymes for reverse transcription enabled the preparation of cDNA clones from plant RNA viruses and opened a broad potential for their use in basic and applied research in the early nineties of the 20th century (Baltimore, 1970; Temin and Mizutani, 1970). This technology allowed us to study the viruses present in a very limited concentration in the host plants or the viruses difficult to isolate. The infectious clones offer valuable information about viral functional genomics, are helpful at the expression and replication studies of RNA viruses using *in vitro* mutagenesis (deletions, insertions, substitutions) and complementation, as well as in the investigation of natural or induced recombination, mechanisms of defect interfering RNA and satellite RNA genesis (Bujarski and Miller, 1992). They may markedly contribute to the analysis of virus-host interactions (e.g. intercellular transport). The infectious clones may be considered as the pools of viral genes for the development of antiviral strategies. Last, but not least, they are an essential source of a material for preparation of the new viral vectors (Boyer and Haenni, 1994).

Usually, a procaryotic system is used for the preparation of infectious clones – the viral genomes are cloned in bacterial plasmids for simple manipulation and application of a standardized laboratory technique, as well as for the multiplication of obtained constructs in a preparative scale. Some complications may occur that arise by the instability of cloned cDNA in *Escherichia coli*, what lead to the point mutations or larger deletions. The reason is a potential toxicity of particular viral sequences for the bacteria. Sometimes, the problem may be eliminated by the substitution of *E. coli* strain or cloning vector (Boyer and Haenni, 1994). Alternatively, the eukaryotic introns may be inserted into critical genome regions, which inactivate the genes in procaryotic cells and are removed by splicing after transfection of the plant host (López-Moya and García, 2000; Zibert *et al.*, 1990; Duke and Palmenberg, 1989). After successful preparation of the full-length cDNA clone, there are several factors negatively influencing its infectivity.

Essentially, infectious clones of RNA viruses may be divided in two types according the transcription place: infectious RNA (*in vitro* transcripts) and infectious cDNA (based on the transcription *in vivo*). Both approaches differ mainly by employed regulation sequences and each of them has its advantages and disadvantages (Boyer and Haenni, 1994).

3.1.1. Infectious transcripts

A transcription *in vitro* is driven by a strong bacteriophage promoter. The promoters of phages γ (Pm), SP6, T3, and mainly T7 have been used (Melton *et al.*, 1984; Dunn and Studier, 1983). Several strategies have been applied to link the transcription promoter to the start of viral sequence. For example, the „universal“ transcription vectors have been constructed with a restrictase-based cloning site exactly in the position of transcription start (Jobling *et al.*, 1988; Yamaya *et al.*, 1988; Ahlquist *et al.*, 1984). The sequence between the promoter and viral cDNA may be eliminated by a directed mutagenesis or by a primer for the cDNA complementary strain synthesis including the promoter region directly linked to the 5' end of the viral sequence (Weiland and Dreher, 1989). To reach a high infectivity, the real 5' end of the transcript has to be identical or highly similar to the wild-type viral sequence. It happens at the cost of the transcript yield, because direct fusion of the promoter with viral cDNA usually does not correspond to the optimal sequence recognized by RNA polymerases (Boyer and Haenni, 1994).

A critical point in the preparation and application of the infectious RNA is the transcription *in vitro* itself (its standardization regarding the quality and quantity of yield) and manipulations with isolated RNA that is very sensitive to the enzyme degradation. On the other hand, the construct does not have to be introduced into the nucleus by transfection – the transcripts function as a translation-ready mRNA in the cytoplasm.

3.1.2. Infectious cDNA

The cDNA clones are transcribed from the CaMV 35S promoter directly in the plants. Expression of the infectious viral RNA by *in vivo* transcription of cDNA-containing vectors has several advantages. It does not require the *in vitro* transcription and is independent on the viral replication process as well as is less sensitive to RNA degradation (Van Bokhoven *et al.*, 1993). The cDNA clones are stable for a long time *in vitro* in the form of isolated plasmid DNA. On the other hand, the construct has to be introduced into the nucleus to allow the transcription, what decreases the efficiency of some transfection methods (Fig. 1). Problems may arise with the transport of complex viral transcripts into the cytoplasm. Moreover, some AU-rich sequences may be recognized as the introns and subjected to splicing in the nucleus (Gleba *et al.*, 2004).

Production of cDNA clones depends on many factors and therefore, it is not possible to arrange an optimal universal protocol useful for all viruses. For the RNA viruses, the first step is the reverse transcription of viral RNA into ssDNA using a primer binding specifically to the 3' terminus. Choice of the primers for following amplification is based on the known genome sequence of particular virus, especially of its terminal regions. According to the adopted strategy, the primers may include linkers for specific restriction enzymes

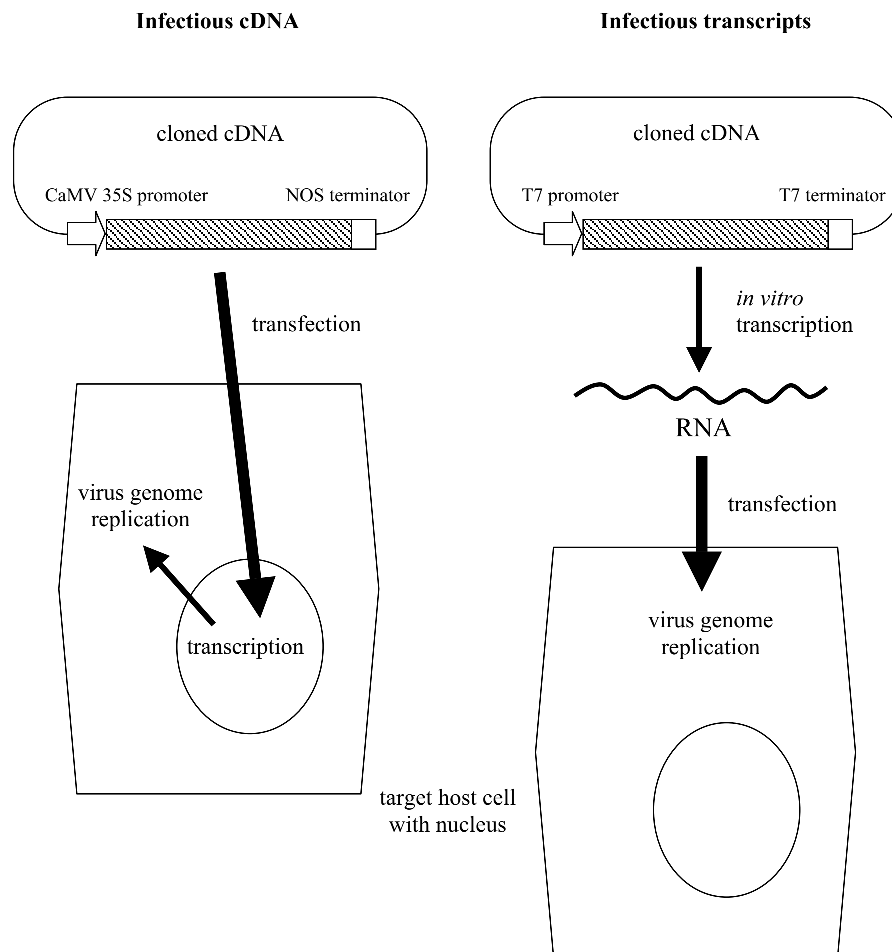

Fig. 1

Comparison of the functions of plant viral cDNA and infectious transcripts

Rectangles with diagonal stripes represent the virus full-length cDNA.

facilitating the cloning process. However, one has to remember that for the successful production of the infectious clone, introducing of any non-viral sequences in the cloning casette between promoter and terminator should be minimized (Boyer and Haenni, 1994). DNA polymerases with a high fidelity are essential for the PCR. The full-length clone may be obtained by a single long PCR that requires special enzymes or by a ligation of several PCR products using natural unique restriction sites.

3.1.3. Transfection of plants

As the final product expression should take place in plants, the prepared clone of viral nucleic acid needs to be introduced into the cells of its potential host. There are several possibilities how to transfect plants.

Throughout agroinfection, the ability of bacteria from the genus *Agrobacterium* to infect plants and to introduce the T-DNA – a part of its tumor-induced plasmid Ti is used. T-DNA is inserted into occasional or at least unpredictable sites of the plant genome (Ziemienowicz *et al.*, 2000). A viral cDNA cloned into T-DNA may be expressed in the plant cells independently on the T-DNA integration into the genome. The agroinfection is especially important for viruses that are difficult to transmitt mechanically (e.g. family *Geminiviridae*). It may be performed *in vitro* with subsequent plant regeneration from a dedifferentiated callus tissue, but also by macroinjection of the agrobacteria into plant stems or by mechanical inoculation of leaves (Liu and Lomonossoff, 2002; Leiser *et al.*, 1992). On the other hand, the agroinfection is not compatible with all plant species, what could be regarded as a disadvantage. This transfection

method is applicable only for the cDNA clones, other methods are suitable for both cDNA and RNA.

In the biolistic method, the nucleic acid molecules are coupled with colloidal gold or tungsten particles, which are subsequently shot directly into plant tissues by a force of compressed helium. There are two types of instruments (gene guns) available – either a box machine (suitable for transfection of protoplasts, tissue cultures, leaf discs, seeds, sprouts or small seedlings in Petri dishes with growth media), or a gun-shaped apparatus, which works also with differentiated tissues of bigger plants in common substrate.

The electroporation is a further transfection method. The solution of a recombinant nucleic acid and plant cells is exposed to a high voltage pulse. It leads to a temporary partial permeabilization of the cell membranes for the exogenous DNA or RNA. This method is applicable mostly for a protoplast transfection, but experiments have been done also with other types of plant cells and tissues (Van Wert and Saunders, 1992).

In the procedure of microinjection, the genes are transferred directly into an isolated cell (protoplast), embryo or meristem culture. The production of chimeric transformed/untransformed plants after a regeneration and differentiation is a disadvantage. The microinjectors are relatively expensive devices and the procedure requires skilled and trained personal (Kost *et al.*, 1995; Reich *et al.*, 1986).

The liposomes can be used for introduction of nucleic acids into protoplasts as well. The advantage is the DNA/RNA stability inside the liposomes, non-invasibility, and reproducibility of the method. However, it is applicable only on the isolated cells (Lurquin and Rollo, 1993).

In the procedure of a mechanical inoculation, the skin of leaves is gently damaged by an abrasive material (carborundum, cellit), what enables the introduction of nucleic acid applied directly on the leaf surface into the cells through the damaged walls as in mechanical transmission of viruses (Hull, 2002).

The transfer of cDNA into the cell nuclei, where the transcription takes place is essential for a successful expression. From this point of view, the most effective methods are the agroinfection, microinjection, and biolistic method (Dagless *et al.*, 1997). The mechanical inoculation is less effective, but very simple, fast, and cheap. Even though only a low percentage of plants are transfected, they may be used as a source of a modified virus in a second round of inoculation, when introduction into nuclei is not required.

3.1.4. Infectivity of prepared clones

The infectivity may be tested and evaluated as the percentage of infected protoplasts, the percentage of individual systemic host plants showing symptoms, the relative number of local lesions in hypersensitive host plants or by the analysis of viral RNA or protein (Commandeur *et al.*, 1991; Hayes and Buck, 1990; Domier *et al.*, 1989; Heaton *et al.*, 1989; Loesch-Fries *et al.*, 1985).

The infectivity of transcripts is variable, in some cases reaching 100 % or more in comparison with the infectivity of parental virion RNA (Hayes and Buck, 1990; Hearne *et al.*, 1990; Sarnow, 1989). Following factors strongly influence the infectivity:

1) Heterogeneity of transcript population relating to the length, especially at the infectious RNA (Hamilton and Baulcombe, 1989; Janda *et al.*, 1987; Dawson *et al.*, 1986; Ahlquist *et al.*, 1984).

2) Presence of point mutations caused by the inaccuracy of RNA- and DNA-synthesis enzymes particularly in the long viral genomes. Changes in a viral sequence can be introduced by the manipulations *in vitro* or by the mutations of the original template of virion RNA (Kuhn *et al.*, 1990). Taking into account the relatively low fidelity of viral RNA-dependent RNA polymerase, the mutations never can be fully eliminated (Ramirez *et al.*, 1995; Domingo and Holland, 1992).

3) The sequences close to the 5' and 3' termini of the construct as the number and sequence of non-viral nucleotides, presence of the cap structure at the 5' terminus or poly (A) tail at the 3' terminus.

3.1.5. Influence of non-viral sequences

Preparation of the infectious clones requires an integration of viral genomes into plant regulatory elements. Therefore, it is impossible to avoid completely insertion of non-viral sequences close to the genome termini. Generally, influence of such sequences close to the 5' end of the viral genome is considerably higher than behind the 3' end.

Sometimes, the infectivity is significantly lowered or eliminated due to the presence of one or two nucleotides (usually G) at the 5' end of the construct. The expression may be inhibited by various mechanisms, but no details are known (Eggen *et al.*, 1989; Janda *et al.*, 1987).

The cap structure on the 5' termini of eucaryotic transcripts is essential for their stability and optimal translation initiation (Green *et al.*, 1983; Contreras *et al.*, 1982; Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977; Shih *et al.*, 1976). In some RNA viruses, the 5' terminus of the genome is free or contains a virus-coded protein (VpG) covalently linked instead of the cap. For those viruses, the infectivity of transcripts could be positively influenced by the addition of the cap structure (Veidt *et al.*, 1992; Eggen *et al.*, 1989). It indicates that the cap may at least partially supply some VpG functions.

The length of the non-viral sequence close to the 3' end influences the biological activity of the viral transcripts too (Beck *et al.*, 1990; Hayes and Buck, 1990; Dzianott and Bujarski, 1989). On the other hand, the transcripts of many viruses bearing long additional sequences (>30 nucleotides)

have been infectious, similar to the transcripts polyadenylated by host cell enzymes *in vivo* (Boyer *et al.*, 1993; Sit and Abouhaidar, 1993; Suzuki *et al.*, 1991). Polyadenylation on the 3' end generally enhances the possibility of construct infectivity. The number of the adenosyl residues critical for the infection is limited for the viruses with polyadenylated genome (Holy and Abouhaidar, 1993; Viry *et al.*, 1993).

The non-viral sequences are systematically removed throughout the replication of viral nucleic acid (Viry *et al.*, 1993; Commandeur *et al.*, 1991; Mori *et al.*, 1991). It may be a consequence of the host nuclease activities or viral polymerases specifically recognizing the correct wild-type genome termini. Unlike the terminal extensions, many internal point mutations (introduced accidentally or purposely) stay usually conserved in the progeny. It reflects the non-lethal character of these mutations rather than repair incapability of the system (Weber *et al.*, 1992; Lai *et al.*, 1991; Hearne *et al.*, 1990; Zibert *et al.*, 1990).

3.2. Construction of viral vectors

Preparation of a DNA clone of the viral genome able to replicate and infect appropriate host plants after transfection is a very important step at the viral vector construction. Therefore, a close attention was paid to this subject in the previous text. Subsequently, a suitable cloning site must be incorporated in the infectious clone. The insertion of foreign genes within the genome of plant viruses is performed by the several methods described in following paragraphs (Scholthof *et al.*, 1996).

3.2.1. Gene exchange

The genes for capsid proteins (CP) were exchanged mostly in the initial experiments. However, these vectors used to lose the ability of systemic or even of cell-to-cell movement because the presence CP was essential (Dawson *et al.*, 1989; French *et al.*, 1986). On the other hand, CP exchange vectors based on bipartite DNA of the geminiviruses were successfully constructed, but there were strong construct-size limitations (Stanley, 1993; Hayes *et al.*, 1988). Promising candidates for the gene exchange are genes regulating the virus transmission by natural vectors. It would result in the disabling of the virus horizontal spread to other than experimentally infected plants (Scholthof *et al.*, 1996). Vectors based on satellite RNA or DNA may be used too (Lin *et al.*, 1996). Some satellite nucleic acids code for non-essential, non-structural proteins, which genes may be replaced by the foreign sequences. They replicate very efficiently and higher expression of the inserted gene can be reached. Satellite DNA of the Tobacco curly shoot virus has been adapted to a vector for silencing of plant genes (Qian *et al.*, 2006).

3.2.2. Gene insertion

There are various possibilities to insert genes into genomes of particular viruses. In the geminiviruses, only small intergenic regions may be used for the insertion. A vector based on the viruses with an icosahedral structure has more stringent requirement concerning the size of nucleic acid than the elongated viruses. In potyviruses, the reading frame must be unbroken and the foreign protein may be expressed either in fusion with some viral products or a new recognition site for viral protease must be constructed for its individual production. Relatively large inserts are tolerated e.g. by the vectors based on the Potato virus X (PVX) (Chapman *et al.*, 1992).

3.2.3. Epitope presentation (CP fusion)

The preparation of epitope presentation systems is a specific application of inserted sequences. It is possible to implement sequences for specific antigen determinants in fusion with the CP in the plant virus genome enabling their exposition on the surface of virus-like particles (Walmsley and Arntzen, 2000). The vectors based on TMV, Cowpea mosaic virus (CPMV) and some other viruses have been adopted for this purpose. In particular, TMV replicates to a high titer in its host and at the same time is easily mechanically transmissible.

3.2.4. Complementation systems

Gene exchange may influence some essential functions of the construct and gene insertion enhances its length, what may lower the encapsidation efficiency. To overcome these problems, it is possible to use so-called complementation systems. A foreign gene is inserted into a defect viral genome, whose replication is dependent on a transgenically inserted viral gene in plants or on a co-infection by a helper virus. Obviously, the complementation by a virus is required also by vectors based on satellite viruses or by nucleic acids (Scholthof *et al.*, 1996).

3.3. Application in research of viral and plant genomes

Prepared viral vectors are usually tested by the expression of well-analyzable reporter genes in transfected plants. Genes for green, eventually cyan or yellow fluorescent proteins (CFP, YFP) are mostly applied (Perez *et al.*, 2006; Bendahmane *et al.*, 2002; Masuta *et al.*, 2000). The infectious clones are used to study the life cycle of viruses, functions of their proteins, and interactions with different host factors, respectively, e.g. the operation mechanisms of resistance genes against respective viruses.

Kim and Palukaitis (1997) studied the target sites of various types of resistance against Cucumber mosaic virus

(CMV) in its genome. The mutations in two sites in the CMV 2a polymerase gene were introduced and the break of resistance by a systemic infection as well as the enhanced accumulation of the 2a protein and viral RNA in protoplasts was achieved. The change of only one of these sites resulted in a systemic infection with a systemic hypersensitive reaction and accumulation of viral RNA in protoplasts. The hypersensitive reaction and localization of the infection was controlled by the separate plant genes.

Bendahmane *et al.* (2002) analyzed the mechanism of action of the TMV CP gene in transgenic plants causing resistance against TMV and related tobamoviruses. For the infection of different transgenic and non-transgenic plants, they used wild-type RNA transcripts or mutated CP gene and found that the standard CP positively influenced the viral movement protein production, while the modified CP had negative effect on its accumulation and function. It led to the increased resistance of plants with introduced mutated CP gene. In this work, a reciprocal regulation of particular virus genes was demonstrated in the infected plant.

A combination of more or less related viruses (construction of chimeric genomes) may provide remarkable facts about functions of the viral genes. Prüfer *et al.* (1995) demonstrated the biological activity of RNA transcripts, as well as cDNA clones of the Cucurbit aphid-borne yellowing virus by their replication abilities in protoplasts and agroinoculated plants. Subsequently, they studied the specifity of luteovirus RNA coating during the virion assembly. Following substitution of the CP gene by the Potato leafroll virus (PLRV) gene, the mixed genome was able of an autonomous replication and virion production.

Paalme *et al.* (2004) combined the genomes of two Potato virus A (PVA) isolates with highly homologous primary structure that differed by their biological properties. The infectivity of prepared chimeras was tested on the *Nicotiana benthamiana* plants. Although the original PVA isolates showed very similar symptoms in this host, one of the constructs did not multiply at all and another one showed a new symptomatogy. Thus, recombination of nearly identical genotypes leads to the generation of a new phenotype. The authors also demonstrated that different parts of potyvirus genome act coordinately.

Perez *et al.* (2006) demonstrated the possibility to construct chimeric genomes from the PVX infectious clones by the method based on the amplification using long primers (so-called megaprimers). This method should be a general and broadly applicable alternative to the common restriction/ligation cloning protocols. By this method, the exchange of two marker genes (CFP and YFP) was performed very efficiently considering the correct clone production and its infectivity.

Liang *et al.* (2004) used a homologous recombination system in yeast for the mutation generation in the PLRV genome. A special developed shuttle vector enabled the mutants and chimeras created in yeast to be transferred into *E. coli* for analysis and confirmation of mutations, as well as into *Agrobacterium tumefaciens* for plant agroinfection. The results showed the good efficiency of the method – high frequency of regenerated mutants, ability to create more than 20 mutants and chimeras by only 2 restriction sites, and the ability to transfer many sequences using only 3–4 DNA fragments.

A binary vector based on the PVX sequence was applied in the screening of *Cladosporum fulvum* cDNA library for the factors invoking hypersensitive reaction in the tomato plants. Use of this expression system in analyses of avirulence factors for other pathogens may be expected (Takken *et al.*, 2000).

Expression of plant genome fragments cloned in viral vectors can lead to a systemic suppression of gene expression by the posttrascriptional gene silencing. In this way, virus-induced gene silencing (VIGS) may be used for analyses of plant gene functions (Lindbo *et al.*, 2001). The VIGS vectors have obvious advantage in comparison to gene silencing by transgenosis, namely their versatility and time- and labor-saving protocols. However, viruses themselves cause some changes in their host gene expression and therefore, one must differ between the influence of the viral vector and the cloned plant gene homologue (Robertson, 2004). The genes conferring a disease and herbicide resistance could be identified and new potential herbicide target genes could be found by application of the VIGS vectors (Lu *et al.*, 2003; Pogue *et al.*, 2002).

3.4. Large-scale production of recombinant proteins in plants

The main goal of plant genetic modifications is to change the properties of a crop in a way leading to increased yields or higher quality of the agricultural products. They are focused mainly on the production of plants resistant to the pests, to herbicides used for weed elimination, and to negative abiotic environmental factors. Another scope of transgenic plants preparation is a preparative production of different foreign substances of protein nature, e.g. hormones, enzymes, antibodies and antigens for vaccination. Specifically for this transgenic plant utilization, a transient expression from viral vectors may be an advantageous alternative.

Essentially, two types of virus vector systems have been generated. The first one is for a presentation of isolated epitopes and the second one for an expression of whole polypeptides (Fig. 2). The vectors based on tobamo-, potex-, poty-, comoviruses, and other taxa were used for these systems (Cañizares *et al.*, 2005). The main advantage is the small size of viral genomes, high yield of the products, and simple manipulation (the cloning and plant transfection by virus vectors is faster and simpler than regeneration of stable lines of transgenic plants). Potential risk of the spread of

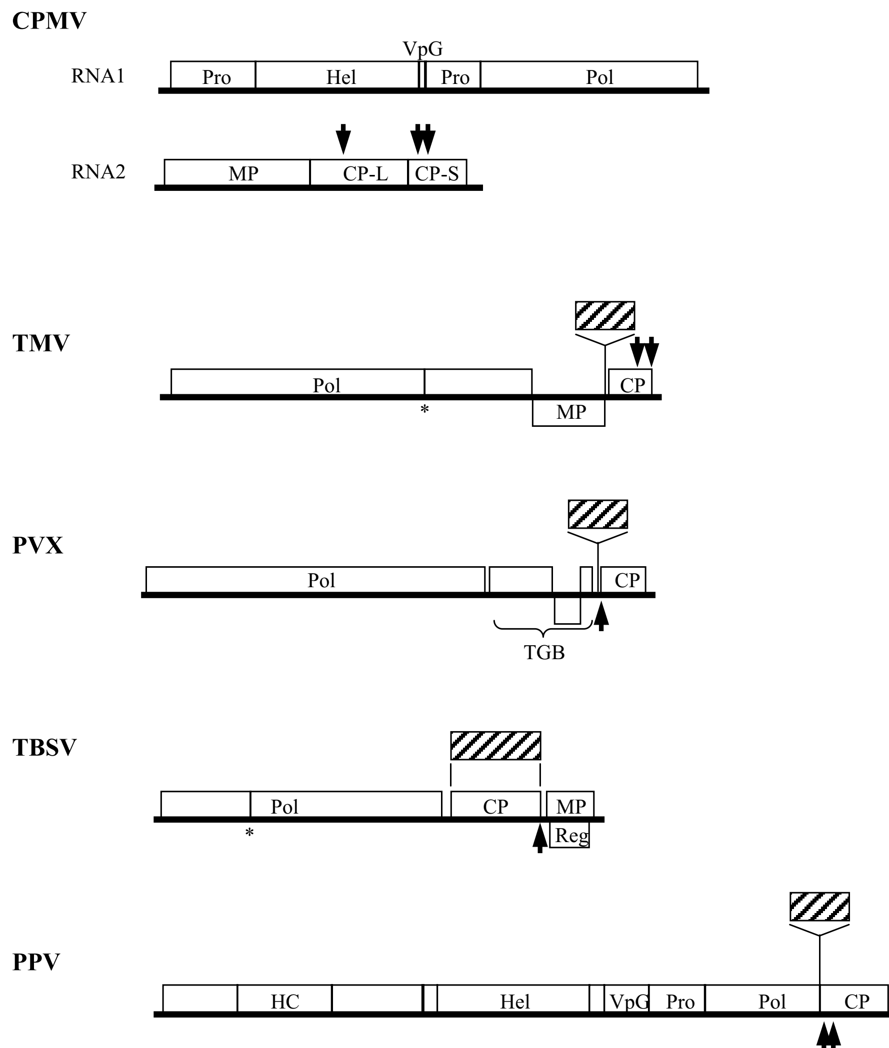

Fig. 2

Schemes of the important vectors based on plant ssRNA viruses

Insertion sites for the foreign fragments are presented as diagonally striped rectangles and epitopes as arrows. Pro = protease; Hel = helicase; Pol = polymerase; TGB = triple gen block; Reg = regulatory protein; HC = helper component for vector transmission. Adopted from Cañizares *et al.* (2005).

modified viruses in the environment is low and desired plant phenotype is not heritable. However, the size and complexity of inserted sequences are limited.

The complete foreign genes are inserted in the viral genome, what leads to the effective polypeptide expression in infected cells usually without fusion. The expressed products of biopharmaceutical value include single-chain antibodies, human α-galactosidase or plant allergens for the application in toleration therapy. Selected published applications and citations are summarized in the Table 1.

A vaccine preparation by the means of the foreign epitope presentation in virus particles has several advantages. The short peptide itself is often unable to initiate production of antibodies, while the purified viral particles induce the antigenic response properly having an adjuvant effect (Savelyeva *et al.*, 2001). Standard virus purification protocols work fine for the virus-like particles, what makes the vaccine preparation fast and cheap. The problems with product expression and stability may occur for particular secondary or tertiary polypeptide structures. A foreign transmembrane domain fused to the CP inhibited systemic movement and caused local necrotic reaction in plants (Li *et al.*, 2006). The effect of a cysteine residue present in the foreign peptide fused to the CP was proved on the morphology and *in vitro* stability of TMV-like particles (Li *et al.*, 2007).

Table 1. Selected applications of plant viruses for the expression of biologically active polypeptides (excluding antigens for immunization)

Vector	Expressed protein	References
TMV	α-trichosanthin	Kumagai *et al*. (1993)
	human α-galactosidase A	Pogue *et al*. (1998)
	tumor-derived scFv vaccine for lymphoma	McCormick *et al*. (1999)
	main birch pollen allergen Bet v1	Krebitz *et al*. (2000)
	Hev b1 and Hev b3 latex allergens	Breiteneder *et al.* (2001)
	bovine follicle stimulating hormone	Dirnberger *et al.* (2001)
	human growth hormone	Gils *et al*. (2005)
ZYMV	*bar* gene product (herbicide resistance)	Shiboleth *et al*. (2001)
	human α-interferon	Arazi *et al*. (2001)
PVX	Bt Cry1Ac toxin and WIN3 proteinase inhibitor (insecticidal proteins)	Lawrence and Novak (2001)
	scFv to TSWV	Franconi *et al*. (1999)
	human granulocyte macrophage colony-stimulating factor	Zhou *et al*. (2006)
CMV	acidic fibroblast growth factor	Matsuo *et al*. (2007)
BPMV	*bar* gene product (herbicide resistance	Zhang and Ghabrial (2006)

ZYMV = Zucchini yellow mosaic virus, TSWV = Tomato spotted wilt virus, BPMV = Bean pod mottle virus.

In addition, a polypeptide expression has been applied for soluble or aggregated antigen preparation. Although the purification process may be more complicated for accumulated heterologous proteins than for virus-like particles, these systems may be implemented for preparation of the immunogens in the plants used as food for the animals. The „edible vaccines" stimulate the mucosal immune system and make a use of the sufficient antigen stability at extremely low pH. They have been generated through viral vectors, although plant transgenosis was the predominating approach including first applications in the human medicine (Lal *et al.*, 2007; Streatfield and Howard, 2003; Walmsley and Arntzen, 2003; Tacket *et al.*, 1998). The application of viruses for the production of vaccines in plants was excellently reviewed (Cañizares *et al.*, 2005; Grill *et al.*, 2005).

CPMV was the first plant virus applied as an epitope presentation system. In most cases, the foreign sequence was inserted in several loops exposed on the virus surface, mostly in the smaller, but also in the larger CP subunit. The prepared chimeric particles were able to induce protective immunity (Lomonossoff and Hamilton, 1999).

TMV particles comprise high number of CP subunits and consequently, this virus is very attractive for peptide expression. A TMV vector was constructed by the inserting a sequence that overreached the CP gene stop-codon and enabled the synthesis of both native and recombinant CP forms from the same viral RNA. This vector was applied e.g. for production of vaccines against the murine herpesvirus or against the Foot and mouth disease virus (Table 2).

To overcome the insert-size limitation, a modified CP of the Alfalfa mosaic virus (AMV) was expressed from the subgenomic TMV promoter. This approach enabled an expression of 40 aa peptide of the Rabies virus or 47 aa peptide of the Human immunodeficiency virus structural proteins fused to the AMV CP. Both particle types induced the production of virus-neutralizing antibodies (Yusibov *et al.*, 1997). Several other viruses as Tomato bushy stunt virus, Plum pox virus or PVX were used for presentation of different antigens (Fig. 2, Table 2).

McCormick *et al.* (2006) were able to stop the myeloma protein production in mice using subcutaneous vaccination by TMV-like particles presenting specific T-cell epitopes. Hence, *in vivo* immunostimulation broadens the potential for application of plant virus vectors.

3.5. Prospective strategies of viral vectors construction

Recently, the epitope presentation system was constructed using sequences of two unrelated plant viruses providing structural and non-structural proteins. CMV CP was expressed from a PVX-based vector and production of CMV virus-like particles in the infected plants was observed. Several neutralizing epitopes of the Newcastle disease virus were successfully engineered on the surface of these particles (Natilla *et al.*, 2006).

In addition, several attempts were performed to insert the complete viral replicon into the plant genome. This approach could overcome such problems as asynchronous expression process and non-uniform product localization in the infected plants. To increase the expression level, a crossing with plants bearing a gene silencing suppressor was demanded (Mallory *et al.*, 2002). Regulated systems are required for the expression of proteins potentially toxic

Table 2. Selected applications of plant viruses for the vaccine preparation by whole polypeptide expression (P) or epitope presentation (EP)

Vector	Target antigen	Vector strategy	References
TMV	VP1 protein of FMDV	P	Wigdorovitz *et al.* (1999)
	gDc protein of BHV-1	P	Perez-Filgueira *et al.* (2003)
	tat protein of HIV-1	P	Karasev *et al.* (2005)
	S glycoprotein of MHV	EP	Koo *et al.* (1999)
	structural peptides of Rabies virus	EP	Yusibov *et al.* (1997)
	D2EIII protein of Dengue virus	P	Saejung *et al.* (2007)
	Ag85B and ESAT6 proteins of *Bacillus tuberculosis*	P	Dorokhov *et al.* (2007)
	L1 protein of HPV-16	P	Varsani *et al.* (2006)
PVX	E7 protein of HPV-16	P	Franconi *et al.* (2002)
	ESAT-6 antigen of the *Bacillus tuberculosis*	P	Zelada *et al.* (2006)
	nucleocapsid protein of HBV	P	Mechtcheriakova *et al.* (2006)
	fibronectin-binding protein of *Staphylococcus aureus*	EP	Brennan *et al.* (1999a)
	E2 glycoprotein of CSFV	EP	Marconi *et al.* (2006)
	neutralizing epitopes of NDV fused with CMV CP	EP	Natilla *et al.* (2006)
PPV	CP2 protein of CPV	EP	Fernández-Fernández *et al.* (1998)
	VP60 protein of RHDV	P	Fernández-Fernández *et al.* (2001)
CPMV	small immunogenic proteins of TGEV	P	Cañizares *et al.* (2005)
	nucleocapsid protein of HBV	P	Mechtcheriakova *et al.* (2006)
	gp41 protein of HIV-1	EP	Porta *et al.* (1994); McLain *et al.* (1996); McInerney *et al.* (1999)
	membrane protein F of *Pseudomonas aeruginosa*	EP	Brennan *et al.* (1999b)
	CP2 protein of CPV	EP	Nicholas *et al.* (2002)
	VP1 protein of FMDV	EP	Usha *et al.* (1993)
TBSV	gp120 glycoprotein of HIV-1	EP	Joelson *et al.* (1997)
	p24 protein of HIV-1	P	Zhang *et al.* (2000)

FMDV = Foot and mouth disease virus, BHV = bovine herpesvirus, HIV-1 = Human immunodefficiency virus 1, MHV = Murine hepatitis virus, HPV = Human papillomavirus, HBV = Hepatitis B virus, RHDV = Rabbit hemorrhagic disease virus, TGEV = Transmissible gastroenteritis virus, PPV = Plum pox virus, CPV = Canine parvovirus, CSFV = Classical svine fever virus, NDV = Newcastle disease virus, TBSV = Tomato bushy stunt virus.

to the plants. This regulation may be mediated by an inducible promoter or by a timed joining of the gene and promotor via induced site-specific recombination (Mor *et al.*, 2003; Mori *et al.*, 2001).

Many of the recently published new approaches for the plant virus vector applications combine them with the plant transgenosis strategy. Jia *et al.* (2006) used a TMV-based vector expressing Cre recombinase for the elimination of *nptII* selection marker gene from the prepared transgenic plants. Hull *et al.* (2005) could induce the transgene expression by infection with an activator-coding TMV vector.

In addition to conventional full viral vectors, a complementation or so called „deconstructed virus“ strategies have been elaborated (Gleba *et al.*, 2004). A viral genome may be divided into particular essential genes for replication, movement, silencing suppression and may be delivered into plants separately using agroinfection or transgenosis. They function in the trans-mode and do not need to be coded by the expression vector. Potentially, such combined integrated systems may be adopted to the plant species that are poor hosts or even non-hosts for particular viruses. The human growth hormone was effectively produced in *N. benthamiana* apoplast by a TMV deconstructed system reaching 10% of total soluble protein (Gils *et al.*, 2005). The bipartite CPMV vector could mediate a high-level expression induced by the transient RNA1 infection of plants previously transformed with the full or partial RNA2 harboring an exogenous gene (Cañizares *et al.*, 2006).

4. Conclusions

The huge improvement of biotechnologies enabled goal-directed *in vivo* preparation of many important biomedicinal substances. Basic requirements for the bioproductions are biological activity, purity, and high yield. To achieve biologically active products, they have to be frequently produced in eucaryotic cells. In comparison to mammalian, insect, and yeast cell bioreactors, the production of biologically active products in plants is much cheaper and simpler. In plants, the proper glycosylation of exogenous proteins was observed (Dirnberger *et al.*, 2001). An attractive alternative to the transgenic approaches is the construction of versatile plant virus vectors for the efficient transient

expression. Following initial infection by either cDNA or RNA, they act as the modified autonomous viruses with high replication rate.

Additionally, they are easily purifiable and storable. The various systems have been laboratory tested for either protein production or screening and function studies of viral and plant genes. The expression of exogenous products may reach the level of viral CP, in order g/kg of fresh tissue mass. A technique for highly effective large-scale field inoculation of plants by high-pressure spraying has been developed (Pogue *et al.*, 2002). A care must be taken considering the potential environmental risk. Nevertheless, according to the actual knowledge its level is very low. Thus, a wide extension of the technologies based on the plant virus vectors may be expected in the near future.

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