Chapter 10
Plant Virus-Mediated Expression in Molecular Farming

Aiming Wang

Abstract Plant viruses have contrasting abilities. On one hand, they can induce gene silencing, termed as virus-induced gene silencing. On the other hand, they have evolved mechanisms that suppress gene silencing and allow the accumulation of very high levels of viral proteins in infected plants. The latter is the driving force for the manipulation of plant viruses for molecular farming in plants. In comparison to the transgenic approach which is often associated with low levels of expression and the requirement of a time-consuming and labour-intensive genetic transformation process, the plant virus-mediated expression approach has several advantages such as easy manipulation, high yield and fast manufacturing. This approach uses plant virus-based expression vectors as a vehicle to produce therapeutic proteins such as antibodies, enzymes, vaccines, and other recombinant proteins of interest in plants. Over the last two decades, a number of plant viruses have been developed and optimized for expression of a variety of pharmaceutical proteins. Some of these recombinant proteins are currently under pre-clinical or clinical trials. In this chapter, I will summarize recent progress, current challenges and future prospects of plant virus-mediated expression in molecular farming.

10.1 Introduction

Since the discovery of the first plant virus, *Tobacco mosaic virus* (TMV), in 1898, virology has become a subject of science (Levine 2001). Over the last century, particularly since the 1980s, plant virology has contributed enormously to the

A. Wang (✉)
Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford St., London, ON N5V 4T3, Canada
e-mail: Aiming.Wang@AGR.GC.CA

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understanding of the fundamental concepts in modern biology as well as the advancement of modern plant biotechnology. Plant viral elements such as promoters, terminators, translational enhancers, and gene silencing suppressors have been extensively studied and widely used in plant biotechnology (Hull 2002). Over the last two decades, plant viruses or their modified versions have been directly employed to drive the transient expression of recombinant proteins in plants (Lomonossoff and Porta 2001; Gleba et al. 2007; Lindbo 2007; Sainsbury et al. 2010a).

As a powerful and versatile platform technology for the expression of recombinant proteins in plants, plant virus-mediated expression systems are superior to the transgenic approach. The foreign proteins encoded by transgenes usually do not accumulate to high levels *in planta*, which bottlenecks the application of the transgenic approach in molecular farming (Doran 2006). However, plant viruses have evolved mechanisms that overcome the plant innate anti-foreign RNA system, e.g., posttranscriptional or virus-induced gene silencing, to overpower host cells to produce large quantities of viral proteins, and to prevent protein degradation with a yet unknown mechanism, allowing for accumulation of large amounts of the viral genome and the viral proteins (Baulcombe 2000). Thus, plant viruses may be manipulated to produce and accumulate large amounts of recombinant proteins within a short period of time. This is significantly superior to the lengthy process of generation and characterization of transgenic plants. Since the turnaround time for expression test is short, the plant viral vector system may be optimized through screening for high expression levels, suitable crop species, and proper protein antigenic sites. Toxic proteins are also less problematic for this system because healthy plants at proper growth stages can be selected for inoculation. Therefore, this system is fast, easy to manipulate, free from chromosomal position effects, highly efficient, and flexible with target proteins and plant species. At early stages, the "full virus" vector strategy was employed to construct the first-generation viral vectors (Gleba et al. 2004, 2007). Essentially the viral vector is an infectious clone that contains the full-length cDNA of the wild-type virus. The second-genera vectors are engineered by the "deconstructed virus" vector strategy. As the deletion versions of the full-length viral vectors, these vectors eliminate undesired or limiting viral genes but retain speed and high productivity (Gleba et al. 2004, 2007).

To date, a number of plant RNA viruses have been developed as powerful and versatile expression vectors for the production of a wide range of heterologous proteins in plants. The most commonly used RNA viruses include TMV, *Potato virus X* (PVX), *Cowpea mosaic virus* (CPMV), and a number of potyviruses. In fact, most of the success in virus-mediated expression in molecular farming has been made using RNA viruses, although breakthroughs in the use of DNA viruses have also been documented recently. As several aspects of this research area have been extensively discussed in a few excellent recent reviews (Lico et al. 2008; De Muynck et al. 2010; Pogue et al. 2010; Rybicki 2010; Gleba and Giritch 2011; other chapters in this book), I will outline in this chapter the most commonly studied plant viruses as expression systems in molecular farming and further briefly summarize recent progress, current challenges and future prospects of plant virus-mediated expression.

10.2 DNA Virus-Mediated Expression

10.2.1 Cauliflower Mosaic Virus

The earliest plant virus expression system for the production of foreign proteins in plants was developed not based on plant RNA viruses but on a DNA virus, *Cauliflower mosaic virus* (CaMV) (Brisson et al. 1984; Fütterer et al. 1990). As the first plant virus was found using DNA instead of RNA as genetic material, CaMV has played an essential and unique role in the fundamental research of plant molecular biology and biotechnology. Its 35S promoter has been widely used as a strong, constitutive expression promoter for various plant research projects as well as commercial applications (Scholthof et al. 1996; Haas et al. 2002). CaMV is the type member of the genus *Caulimovirus* in the family *Caulimoviridae*. The icosahedral virions are non-enveloped isometric particles with 420 coat protein (CP) subunits. The viral genome is a double-stranded circular DNA molecule of approximately 8 kb. It has two intergenic regions of regulation and six major open reading frames (ORFs). Its genome replication is through reverse transcription of a pregenomic RNA. Brisson et al. (1984) replaced an aphid transmission factor domain in the ORF II with a bacterial dihydrofolate reductase (DHFR) that confers resistance to methotrexate. The resulting vector was used to inoculate turnip leaves and the recombinant virus spread systemically in the inoculated plants. Typical CaMV symptoms were observed. The plant-derived DHFR directed by CaMV was biologically active (Brisson et al. 1984). The CaMV vector was also successfully used for the expression of a Chinese hamster metallothionein (CHMT II) and a human interferon in turnip plants (Lefebvre et al. 1987; de Zoeten et al. 1989). CaMV showed some potential as the recombinant protein could reach 0.5% of the total soluble leaf protein (Lefebvre et al. 1987). It was quickly found out that the use of the CaMV vector is hampered by the narrow range of plants (limited to the *Cruciferae* family and a few species in the family *Solanaceae*) infected by CaMV, and by practical limitations on inserting foreign DNA that are imposed by the biology of CaMV (Fütterer et al. 1990; Scholthof et al. 1996; Haas et al. 2002).

10.2.2 Geminiviruses

Viruses in another DNA virus family *Geminiviridae* have also been developed as vectors for the production of proteins of interest in plants. The viral genome of this family consists of one or two circular single-stranded DNA (ssDNA) molecules of 2.5–3.0 kb in length (Gutirrez 1999, 2000). Based on genomic organization, host range and insect vectors, viruses in this family are classified into four genera, e.g., *Mastrevirus*, *Begomovirus*, *Curtovirus*, and *Topocuvirus*. Although viruses in all the four genera have been studied as potential expression vectors, excellent progress has been made from several viruses in the first two genera.

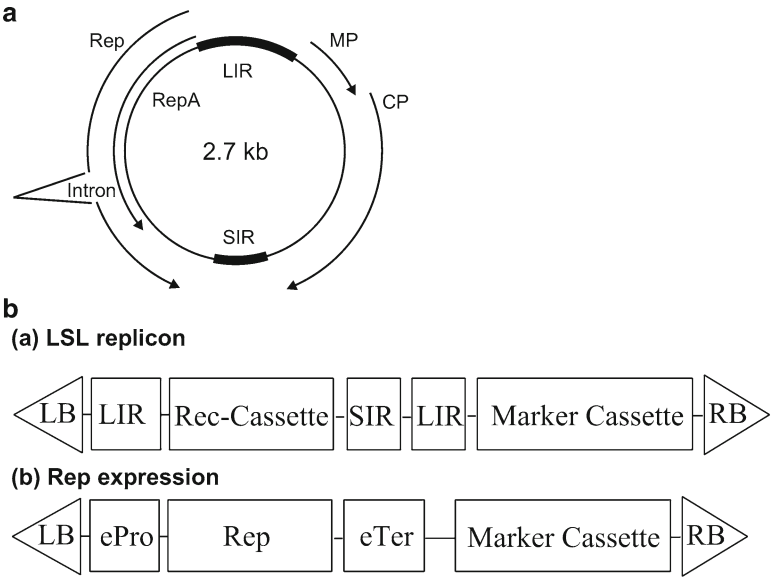

Fig. 10.1 Geminivirus-mediated expression in molecular farming. (**a**) Genomic organization of mastreviruses including *Bean yellow dwarf virus* (BeYDV). (**b**) The BeYDV-based dual-vector system for the high-level expression of recombinant proteins in plants. *CP* coat protein, *ePro* plant eukaryotic promoter, *eTer* plant eukaryotic terminator, *IR* intergenic region, *LB* left boarder, *LIR* large intergenic region, *Marker cassette* marker gene expression cassette, *MP* movement protein, *RB* right boarder, *Rec-Cassette* recombinant protein expression cassette, *Rep* replicase, *RepA* resulting from translation of the differentially spliced transcript

The leafhopper-transmitted mastreviruses including *Bean yellow dwarf virus* (BeYDV), *Maize streak virus* (MSV), *Wheat dwarf virus* (WDV), and *Tobacco yellow dwarf virus* (TYDV) have a single genome component of about 2.7 kb (Needham et al. 1998; Hefferon and Fan 2004; Hefferon et al. 2004; Huang et al. 2009; Regnard et al. 2010). Most mastreviruses are confined to monocotyledonous plants, but some of them such as BeYDV and TYDV infect dicots. The mastreviral genome has a long intergenic region (LIR) that consists of transcriptional promoters and the viral origin of replication, and a short intergenic region (SIR) that contains transcription termination signals and the DNA primer binding site for complementary strand DNA synthesis (Fig. 10.1) (Regnard et al. 2010). Similar to other geminiviruses, mastreviruses carry out their genome replication in the nuclei of the infected cells using a rolling circle mechanism (Gutierrez 1999, 2000). During genome replication, numerous double-stranded DNA replicative form intermediates are produced for both replication and transcription. Mastreviruses have only three genes: the viral sense genes *V1* and *V2* encoding the movement protein (MP) and the coat protein (CP), respectively and the complementary sense gene *Rep* coding for two replicase proteins, Rep and RepA (resulting from an alternative splicing) (Fig. 10.1). Early endeavors were concentrated on MSV and WDV (Laufs et al. 1990;

Matzeit et al. 1991; Ugaki et al. 1991; Timmermans et al. 1992, 1994). In those MSV or WDV-derived vectors, MP or CP was replaced by foreign proteins, and infection and replication were limited within protoplasts or primarily infected cells. An improved MSV-derived vector where foreign genes were inserted into the non-coding region of the viral genome was shown to be able to systematically infect maize plants with an increased level of recombinant protein accumulation (Shen and Hohn 1994, 1995). Unfortunately, the overall foreign protein yield was not satisfactory.

Recent focus has shifted on dicot-infecting mastreviruses such as BeYDV (Mor et al. 2003; Zhang et al. 2006; Huang et al. 2009; Regnard et al. 2010). A BeYDV-derived LSL replicon from a plasmid or a chromosome-integrated transgene could be rescued by the presence of the replication initiation protein, Rep (Fig. 10.1). High-level expression of GUS was found in tobacco NT1 cell suspensions co-transfected with the LSL vector carrying a GUS reporter and a Rep-supplying vector (Mor et al. 2003). In this system, Rep induced release of the BeYDV replicon and episomal replication to high copy number (Mor et al. 2003). This system was tested with two transgenes. One of them was the BeYDV replicon with an expression cassette (allowing for expression of the genes of interest) flanked by *cis*-acting DNA elements of BeYDV. The other was to express Rep under an alcohol-inducible promoter. Both of them were transformed into the tobacco NT1 cells and potato plants. After ethanol treatment, transgene mRNA and protein levels in the NT1 cells and the leaves of whole potato plants increased by 80 and 10 times, respectively (Zhang et al. 2006). To avoid genetic transformation, co-delivery of the BeYDV-derived vector and the Rep/RepA supplying vector was attempted by agroinfiltration of *Nicotinana benthamiana* (Huang et al. 2009) (Fig. 10.1). The improved system largely enhanced recombinant protein accumulation (Huang et al. 2009). More recently, Regnard et al. (2010) developed a new BeYDV vector, pRIC. This vector differs from other BeYDV- and other geminivirus-derived vectors published previously. In this vector system, the BeYDV replicase proteins were included *in cis*. High level protein expression in plants by using this autonomously replicating shuttle vector was reported (Regnard et al. 2010).

In addition to mastreviruses, the whitefly-transmitted begomoviruses, such as *Bean golden mosaic virus* (BGMV), *Tomato gold mosaic virus* (TGMV), *Tomato yellow leaf curl virus* (TYLCV) and *Ageratum yellow vein virus* (AYVV), have also been investigated for their suitability in molecular farming. This group of geminiviruses exclusively infects dicotyledonous plants. Most begomoviruses have a bipartite genome with two ssDNA molecules (DNA A and DNA B) of approximately 2.6 kb each. As early as in 1988, Hayes et al. successfully developed a TGMV-based vector. This vector was used to express bacterial neomycin phosphotransferase (NPT) (gene *neo*) and bacterial β-glucuronidase (GUS) (gene *uidA*) in tobacco (Hayes et al. 1988, 1989). However, this vector seemed unstable as deletion was evident (Hays et al. 1989). A few members of begomoviruses such as AYVV and TYLCV that have a monopartite genome have also been tested as viral expression vectors (Tamilselvi et al. 2004; Perez et al. 2007). Although not much progress has been made so far, such monopartite begomoviruses are predicted to be of great potential

in molecular farming as they are dicotyledonous plant viruses and share the similar genomic structure with BeYDV-like mastreviruses.

10.3 RNA Virus-Mediated Expression

Numerous plant RNA viruses have been explored for their uses in molecular farming. In most cases, the foreign gene is either to replace or to be fused in-frame with CP and the viral cDNA containing the foreign gene is placed under the control of a strong bacteriophage promoter such as SP6, T3 and T7 in an *in vitro* transcription vector or under a eukaryotic constitutive expression promoter such as the CaMV 35S promoter in a binary vector (Fig. 10.2). For the former, infectious transcripts are obtained by *in vitro* transcription and introduced into plants via mechanical inoculation. For the latter, transient expression is achieved by mechanical inoculation, biolistic delivery or agroinfection. Here I will cover the most commonly used plant RNA virus-mediated expression systems.

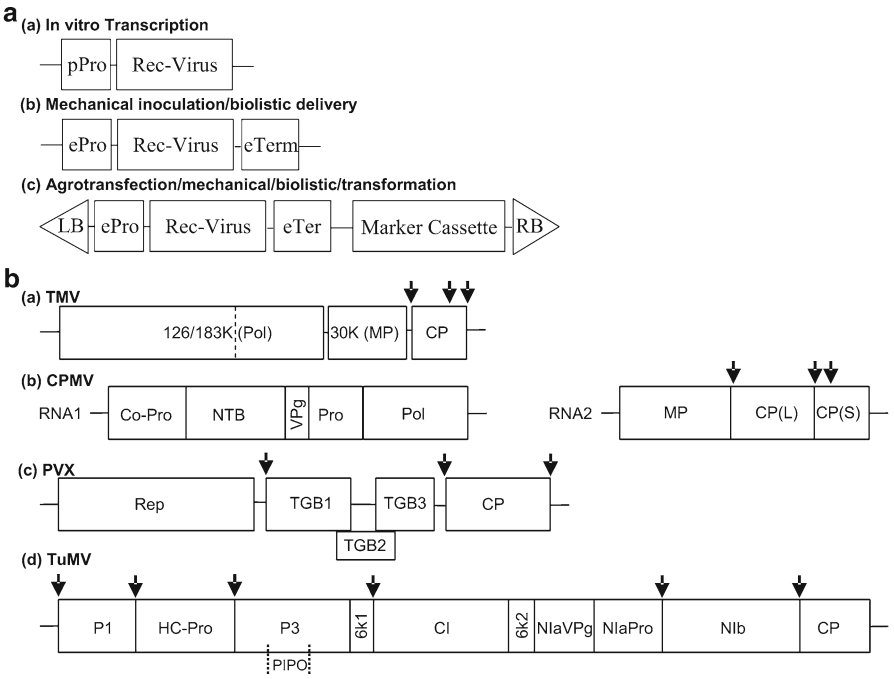

Fig. 10.2 RNA virus-mediated expression in molecular farming. (**a**) Commonly used strategies for vector construction. *pPro* prokaryotic promoter (such as SP6, T3 and T7), *Rec-virus* recombinant virus, *ePro* plant eukaryotic promoter, *eTerm* plant eukaryotic terminator. (**b**) Construction of genomic organization of plant RNA viruses that have been extensively used to express peptides and proteins of interest in plants. *Arrows* point to the positions where foreign proteins are inserted. *TMV* Tobacco mosaic virus, *CPMV* Cowpea mosaic virus, *PVX* Potato virus X, and *TuMV* Turnip mosaic virus

10.3.1 TMV

TMV is the type member of the genus *Tobamovirus*. As the first named virus, TMV is considered the best studied RNA virus. The TMV-based viral vector is also the most commonly used one in molecular farming. TMV has a simple viral genome, which is a 6.4 kb single-stranded positive sense RNA molecule encoding four viral proteins (Fig. 10.2). The first ORF encodes two replicase proteins, 126- and 183-kDa proteins, which are required for viral RNA replication. The 183-kDa replicase results from read-through of the amber stop codon for the 126-kDa protein. The 30-kDa MP and 17-kDa CP are translated via two subgenomic RNAs from the 3'-proximal ORFs. As the 17-kDa CP is the most abundant protein, CP fusion or replacement of CP with the foreign gene to be expressed was the primary strategy for the expression of foreign proteins (Takamatsu et al. 1987). However, this kind of recombinant TMVs was unable to infect systemically due to the lack or disturbance of the functional CP. This problem was solved by cloning the target gene under the control of the TMV CP subgenomic promoter and inserting this expression unit between MP and CP of the viral vector (Kumagai et al. 1993). This strategy was successfully used to express, at high levels, a number of recombinant proteins such as α-trichosanthin (Kumagai et al. 1993), human papillomavirus CP L1 (Varsani et al. 2006), GFP (Lindbo 2007), and human growth hormone (Skarjinskaia et al. 2008) in plants.

10.3.2 CPMV

CPMV, the type member of the genus *Comovirus*, is a bipartite virus consisting of two positive-sense single-stranded RNAs, RNA1 and RNA2 as its genome. Each RNA has a 3'-poly(A) tail and a viral protein genome-linked (VPg) covalently linked at the 5' terminus (Lomonossoff and Porta 2001). RNA1 is about 5.9 kb and RNA2 ~3.5 kb in length, each encoding a single polyprotein that is processed by the RNA1-encoded 24-kDa protease domain (Fig. 10.2). RNA1-encoded proteins are required for viral genome replication and polyprotein processing; RNA2 encodes MP and two CPs, i.e., CP(L) and CP(S). As RNA1 is essential for replication, RNA2 is the target of manipulation. Early work was focused on fusion or replacement of the CP(S) for the expression of epitopes such as an epitope of VP1 of *Foot-and-mouth disease virus* (FDMV) (Usha et al. 1993), epitopes derived from human rhinovirus and human immunodeficiency virus type 1 (HIV-1) (Porta et al. 1994) and several other epitopes (McLain et al. 1995; Dalsgaard et al. 1997; Brennan et al. 1999; Taylor et al. 2000). In addition to epitopes, CPMV has also been used for the high-level expression of a variety of recombinant proteins. Cañizares et al. (2006) reported an improved CPMV expression system. In this system, the chimeric gene coding for the 2A protease of FMDV and the target protein was in frame fused to the C-terminus of the CP(S) of RNA2. Upon translation, the target protein can be released by the 2A protease. Transgenic plants containing the recombinant CPMV RNA2 cDNA

controlled by the *CaMV 35S* promoter was generated. When the transgenic plants derived from this vector were agroinoculated with a plant expression vector to produce RNA1-encoded proteins or genetically crossed with another transgenic plant expressing RNA1, active recombinant CPMV replication would occur, leading to the production of large amounts of recombinant protein. Cañizares et al. (2006) reported a deletion version of RNA2 where the recombinant RNA2 cDNA was precisely deleted to keep the 5' UTR and 3' UTR under the control of the 35S promoter. The target gene can be inserted between the 5' and 3' UTR for expression. More recently, Sainsbury et al. (2008, 2010b) have further modified the CPMV expression system to produce high quality functional anti-HIV antibodies.

10.3.3 PVX

PVX, the type member of the genus *Potexvirus*, is a monopartite virus with a single, positive-stranded RNA molecule of approximately 6.5 kb as its genome (Chapman et al. 1992). The 5' capped, 3' polyadenylated genomic RNA has five ORFs encoding a replicase (Rep), a set of three movement proteins (triple gene block: TGB1, TGB2 and TGB3) and CP (Batten et al. 2003; Avesani et al. 2007) (Fig. 10.2). PVX-mediated expression has been extensively studied for expression of heterologous proteins in plants (Chapman et al. 1992; Baulcombe et al. 1995; Hammond-Kosack et al. 1995; Sablowski et al. 1995; Angell and Baulcombe 1997; Santa et al. 1996; O'Brien et al. 2000; Ziegler et al. 2000; Marusic et al. 2001; Toth et al. 2001; Franconi et al. 2002; Avesani et al. 2003; Čeřovská et al. 2004; Manske et al. 2005; Uhde et al. 2005; Komorova et al. 2006; Ravin et al. 2008). As the recombinant PVX lacking CP (replaced by the target gene) failed to infect plants systemically resulting in poor accumulation of recombinant proteins, this gene replacement strategy seemed ineffective (Chapman et al. 1992). A new strategy, i.e., CP fusions, was proposed and tested for the production of vaccine antigenic sites (Santa et al. 1996; O'Brien et al. 2000; Marusic et al. 2001; Uhde et al. 2005). However, this system requires that the CP-fusion does not compromise viral particle assembly and the systemic infectivity of the recombinant virus. Therefore, it is only suitable for the expression of small protein tags or antigen epitopes. The gene insertion strategy was further developed to overcome this drawback. This system allows for insertion of the target gene into the viral genome coupled to a duplicated copy of the CP promoter (Baulcombe et al. 1995). This improved version infected systemically and directed high-levels of expression of a variety of recombinant proteins such as GFP and the human islet autoantigen glutamic acid decarboxylase (hGAD65) (Baulcombe et al. 1995; Avesani et al. 2003). Recent studies have shown that a minimal PVX vector in which the triple block and CP were removed can allow for high-level expression (Komorova et al. 2006; Ravin et al. 2008). The target gene was inserted downstream of the first viral subgenomic promoter and transcription of the recombinant PVX was controlled by the 35S promoter. In agroinfiltrated plant leaves, the recombinant protein accumulated up to 2% of total soluble proteins (Ravin et al. 2008).

10.3.4 Potyviruses

Potyviruses represent the largest and most agriculturally important plant virus group (Urcuqui-Inchima et al. 2001). The monopartite virus has a single-stranded positive-sense RNA molecular of ~10 kb as its genome. Similar to CPMV, the genomic RNA has a 3'-poly(A) tail and a viral protein genome-linked (VPg) covalently linked at the 5' terminus. The virus adopts a polyprotein strategy. The only long ORF encodes a large polyprotein of ~350 kDa, and also a shorter polyprotein as a result of translational frameshift in the P3 coding region (Fig. 10.2). The two polyproteins are processed by virus-encoded proteases to release 11 mature proteins, from the N-terminus, P1, HC-Pro, P3, P3N-PIPO, 6 K1, CI, 6 K2, NIa-VPg, NIa-Pro, NIb, and CP (Chung et al. 2008). Since recombinant potyviruses can allow for simultaneous equimolecular expression of multiple foreign genes and the lengths of the foreign genes are flexible, a number of potyviruses have been developed as a viral expression vector, including *Turnip mosaic virus* (TuMV), *Tobacco etch virus* (TEV), *Plum pox virus* (PPV), *Lettuce mosaic virus* (LMV), *Clover yellow vein virus* (ClYVV), *Pea seed-borne mosaic virus* (PSbMV), *Potato virus A* (PVA), *Zucchini yellow mosaic virus* (ZYMV) and *Soybean mosaic virus* (SMV) (Dolja et al. 1992; Verchot et al. 1995; Guo et al. 1998; Whitham et al. 1999; German-Retana et al. 2000; Masuta et al. 2000; Johansen et al. 2001; Hsu et al. 2004; Beauchemin et al. 2005; Kelloniemi et al. 2008; Wang et al. 2008). TEV was the first potyvrius-derived vector and the foreign gene was in-frame inserted at the junction of P1 and HC-Pro for expression (Dolja et al. 1992). In addition to the P1/HC-Pro site, other junctions such as P1/HC-Pro, NIa-Pro/NIb and NIb/CP have also been found suitable for the simultaneous expression of several heterologous proteins (Chen et al. 2007; Kelloniemi et al. 2008; Bedoya et al. 2010).

10.4 Virus-Mediated Expression of Antibodies

Antibodies play an important role in several aspects of medical science such as research, therapy and diagnostics. Virus-based expression has been explored for the production of recombinant antibodies in plants to satisfy the growing demand. Verch et al. (1998) pioneered such research by using a TMV-based vector to express monoclonal antibody (mAb) CO17-1A, directed to a colon cancer antigen, in *N. benthamiana*. Two recombinant TMV clones were engineered to express heavy and light chains of this antibody. Plants co-transfected with both recombinant viral constructs expressed the heavy and light chains that were assembled into a biologically active full-length antibody (Verch et al. 1998). To enhance the recombinant antibody yield, Giritch et al. (2006) used two noncompeting viral vectors derived from TMV and PVX, each expressing one different chain of the human tumor-specific mAb A5 independently. The two viral vectors effectively coexpressed the heavy and light chains in the same cell throughout the plant with yields of up to 0.5 g of assembled mAbs per kg of fresh-leaf biomass (Giritch et al. 2006).

The same two-noncompeting viral vector system was successfully used to produce the humanized murine mAb, Hu-E16 (Lai et al. 2010). The plant-derive mAb had therapeutic activity as effective as the mammalian-cell-produced HuE16 against *West Nile virus* (Lai et al. 2010).

10.5 Virus-Mediated Expression of Vaccines

A number of plant-derived candidate vaccines through virus-mediated expression have been produced against the causal pathogen such as a virus, bacterium or parasite (termed prophylactic vaccine) and a disease such as cancer (termed therapeutic vaccine) in humans and animals. About 30 representative plant-based antigens expressed through viral vectors before 2007 were listed in an excellent review by Lico et al. (2008). Based on the nature of the recombinant proteins, these plant-based vaccines can be classified into two types: free proteins (vaccine subunits) and peptide or epitope fusions to viral CP (which forms empty viral particles) or to other proteins (Pogue et al. 2002; Rybicki 2010). Unlike the traditional vaccines that are often injected intraperitoneally for vaccination, plant-derived vaccines either through virus-mediated expression or other expression systems can be administered into the body orally, intranasally or by needle injection, as most of plants are edible (Awram et al. 2002). Oral or nasal administration that can induce specific mucosal immune response may be the best approach against pathogens as the vast majority of pathogens enter the body through the mucosal surface.

10.5.1 Prophylactic Vaccines

The TMV-based vector was used to express the malarial epitope-CP fusion protein, one of the first plant-based vaccines (Turpen et al. 1995). The recombinant TMV technology was modified to express numerous prophylactic and therapeutic vaccines. For instance, the 5B19 epitope of the spike protein of *Murine hepatitis virus* (MHV) was fused to the C-terminus of the CP of a TMV vector. The mice immunized through either subcutaneous or intranasal routes survived challenge with a lethal dose of MHV strain JHM (Koo et al. 1999). In the case of free vaccine subunits, the structural proteins VP1 of FMDV carrying critical epitopes responsible for the induction of neutralizing antibodies was expressed by a TMV-based vector and needle injection of mice with foliar extracts containing VP1 induced immune protection against a lethal FMDV infection (Wigdorovitz et al. 1999). Recently, a new version of the TMV-based vector that was delivered into plants via agroinfiltration has been successfully used to express several antigens against both viral and bacterial pathogens (Musiychuk et al. 2007; Mett et al. 2008; Chichester et al. 2009).

Plant-based vaccines were expressed by other viral vectors too. For instance, a PPV-based vector was employed to express the entire VP60 of *Rabbit hemorrhagic*

disease virus (RHDV) by insertion into the junction of NIb/CP. Immunization of rabbits via the subcutaneous route with protein extracts containing VP60 protected the immunized rabbits against a lethal challenge with RHDV (Fernádez-Fernádez et al. 2001). A PVX-based vector was used to express the CP fusion with a highly conserved ELDKWA epitope from glycoprotein (gp) 41 of HIV-1. Normal or immunodeficient mice were immunized intraperitoneally or intranasally with the purified chimeric particles from *N. benthamiana* leaves inoculated with the recombinant PVX. High levels of HIV-1-specific immune response were found in these mice. Sera from either normal or immunodeficient mice immunized with the plant-derived CP fusions showed an anti-HIV-1 neutralizing activity (Marusic et al. 2001).

10.5.2 Therapeutic Vaccines

The recombinant TMV technology was also used to express therapeutic vaccines against challenging chronic diseases. An excellent example was to express therapeutic vaccines against cancers. B cell tumours express a unique cell surface Ig which is a tumor-specific marker and vaccination of patients with this Ig often achieves a superior clinical outcome (McCormick et al. 1999). An ideotype-specific single-chain Fv fragment (scFv) of the immunoglobulin from the 38 C13 mouse B cell lymphoma was cloned into a TMV vector and expressed in *N. benthamiana*. Mice immunized with the plant-made scFV were protected from challenge by a lethal dose of the syngenetic 38 C13 tumor cells (McCormick et al. 1999). Recently, the same viral expression system has been used to express patient-specific scFVs from individual patient's tumour. Results from a Phase I clinical study by immunization of patients with their own individual therapeutic antigen produced in plants suggest that the ideotype vaccines produced through virus-mediated expression are safe to administer and offer follicular lymphoma patients with a viable option for ideotype-specific immune therapy (McCormick et al. 2008).

10.6 Virus-Mediated Expression of Recombinant Proteins with Other Functions

Plant-produced proteins via virus-mediated expression may have diverse functions such as insecticides and industrial enzymes. For example, a TMV vector was used to produce rice α-amylase in *N. Benthamiana* (Kumagai et al. 2000). The plant-produced enzyme was moderately glycosylated and was accumulated to levels of at least 5% of total soluble protein. A potential larvicide was also produced through TMV-mediated expression in plants (Borovsky et al. 2006). A mosquito decapeptide hormone, the *Aedes aegypti* trypsin-modulating oostatic factor (TMOF) was fused with CP. In *N. tabacum* infected by the recombinant TMV, the TMOF fusion could reach

to levels of 1.3% of total soluble protein. TMOF fusion-expressing tobacco discs effectively inhibited the growth of *Heliothis virescens*. Purified CP-TMOF virions fed to mosquito larvae arrested larval growth and caused death (Borovsky et al. 2006).

10.7 Conclusions

Like other plant production systems, the virus-based expression system has its strengths and weaknesses. There are several challenges that may prevent the application of this system for commercial uses. First, each particular virus-mediated expression system is limited to certain plant species due to the host range of plant viruses. Second, plant virus-mediated expression is associated with the potential high error frequency and rapid recombination (van Vloten-Doting et al. 1985; Drake and Holland 1999). The relatively high mutation rate of the viral RNA-dependent RNA polymerase due to the lack of proofreading ability may result in the production of mixed recombinant proteins containing undesirable proteins. Rapid recombination may cause instability and deletion of the foreign genes (Scholthof et al. 1996; García-Arenal et al. 2003). Third, there are bio- and environmental concerns about the impact of recombinant viruses (Pogue et al. 2002). Although the risk of human and animal infection by exposure in the field or in food products to plant viruses is ruled out due to their non-infectivity to human beings and animals, recombinant plant viruses can spread to weeds or other crops in their host range. Containment measures through physical barriers or biotechnology (mutations in the genes responsible for insect transmission to prevent insect transmission in the field) should be assessed. In addition, other practical issues, such as expression stability, the biological activity of the product, and downstream processing, must be addressed before large-scale commercial uses of the plant viral vector system.

In view of the growing demand of the market for recombinant proteins with diverse functions, virus-mediated expression in plants has proved to be a promising alternative means to satisfy this need. In the past several years, several new vector systems revolutionized the area with improved viral vector stability, high protein yields and low bio- and environmental risks (Sanchez-Navarro et al. 2001; Perez et al. 2007; Huang et al. 2009; Regnard et al. 2010; Sainsbury et al. 2010a). Some systems require just a few weeks from obtaining the DNA sequence of a viral pathogen to the production of candidate vaccine subunits against it. This very short turn-around time also makes “personalized therapeutic proteins” such as antibodies possible and practical. Along with the improvement of virus-mediated expression technologies, the number of virus-derived recombinant proteins is expected to increase significantly. As a result, more and more plant-derived recombinant proteins via virus-mediated expression will move through clinic trials into commercialization in the coming years. This exciting vision will certainly stimulate new investments on research and development of next generation virus-mediated expression systems that will circumvent current technological drawbacks and new challenges emerging during the course of research, production and commercialization.

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