Proceedings of ANAS (Biological Sciences), 65(5-6): 195-200 (2010)

Plants as an Alternative System for Expression of Vaccine Antigens

Vidadi M. Yusibov*, Tarlan G. Mamedov
Fraunhofer USA Center for Molecular Biotechnology, 9 Innovation Way, Suite 200, Newark, DE 19713, USA

As a demand for vaccine production continues to grow, new innovative approaches and cell substrates for vaccine antigen expression are being developed. Recently, an increasing interest in using plants for recombinant protein production, including vaccine antigens and therapeutic antibodies, has been observed. Here, we overview plant-based expression systems and discuss their advantages, including high and most rapid production capacity, relatively low capital investment, and freedom from contamination with animal pathogens.

Keywords: *vaccine, expression system, plant-based transient expression, launch vector*

INTRODUCTION

Over the last several decades, the development of DNA-based technologies and recombinant protein expression systems (both *in vitro* and *in vivo*) has created opportunities for the design and manufacture of new types of vaccines, and has ushered in a renaissance in the field of vaccinology. This was further stimulated by advances in genomics and proteomics which significantly improved our understanding of molecular pathways underlying infectious disease pathogenesis, host immunity, and host-pathogen interactions.

The expression systems that have been developed so far and extensively used to produce various types of therapeutic recombinant proteins include cell cultures of bacterial, yeast, insect and mammalian origin (Rogan and Babiuk, 2005). The bacterial system is cheap and effective in recombinant protein expression, and currently is the most employed one. However, production of eukaryotic proteins in bacteria has serious limitations. Specifically, bacteria are not capable of post-translational modifications, such as phosphorylation and N-linked glycosylation, which significantly affects biological activity, solubility, stability, half-life, and protease resistance of recombinant proteins. Yeast is an excellent eukaryotic host for the production of recombinant proteins due to low cost and scalability. In addition, this production system is safe as yeasts do not harbor mammalian viruses and do not produce toxins that are hazardous to humans. However, both N- and O-linked oligosaccharide structures produced in yeasts are significantly different from their mammalian counterparts, and hypermannosylation, a common feature in yeast, significantly affects proper protein folding and therefore, its functional activity.

A baculovirus-infected insect cell host system offers significant advantages compared to other expression systems, including the ease of cultivation, capacity for high-level target expression, and possession of post-translational protein modification machinery suitable for complex mammalian proteins. The disadvantages of the insect cell system include the exclusively high-mannose type glycosylation and the lack of complex oligosaccharides containing fucose, galactose and terminal sialic acid (Rai and Padh, 2001). In addition, an internal cleavage site in proteins produced in yeasts, which is located within the arginine- or lysine-rich sequence, is extremely inefficient and leads to improper protein processing.

Mammalian cell culture is still considered an ideal platform for expression of recombinant proteins that require mammalian-type post-translational modifications (some complex-structure large-molecule proteins) such as glycosylation, phosphorylation and γ-carboxylation. This system offers the greatest degree of product fidelity; however, this system is extremely expensive and difficult to scale up.

Thus, expression systems that have a high capacity to provide soluble, post-translationally modified (when necessary), and correctly folded proteins are continually being developed (Houdebine et al., 2000; Gasdaska et al., 2003; Rasala et al., 2010). In this paper, we overview several new expression systems, with the major focus on plants, which are being developed in an attempt to address the above concerns.

*E-mail: vyusibov@fraunhofer-cmb.org

MODERN VACCINES

Although vaccination has been practiced for over 200 years, its principle – a prophylaxis against infectious diseases – has not changed. The advances in understanding molecular biology and pathology of disease-causing agents, and in recombinant DNA tools and techniques have opened new opportunities for developing new genetically engineered vaccines, including recombinant protein subunit and DNA-based vaccines. Although the majority of currently licensed vaccines are based on killed or live attenuated pathogens, subunit vaccines that are based on specific antigens or toxins are becoming preferred alternatives due to their safety, efficacy and scalability. Recent examples of recombinant antigen-based vaccines produced in the yeast or insect cell expression system that have been approved for human use are vaccines against Hepatitis B virus or Human papilloma virus (HPV) (Lowy and Schiller, 2006). The increased use of genomics approaches in subunit vaccine development has facilitated identification of several new vaccine candidates. This, in turn, requires expression systems that allow for rapid production of a broad variety of targets that are correctly folded, are safe, and confer pathogen-specific immunity.

The recent biotechnology boom has triggered an interest in utilization of plants as an alternative expression system for production of recombinant proteins. The first plant-derived recombinant protein, human serum albumin, was produced in transgenic tobacco in 1990 (Sijmons et al., 1990). Since then, plants have been used to express a variety of other therapeutic proteins, blood components, cytokines, hormones, growth factors, vaccines and antibodies. This resulted in federal approval (US Department of Agriculture Center for Veterinary Biologics) in 2006 of the first plant-made vaccine against Newcastle disease in poultry developed by Dow AgroSciences LLC (Indianapolis, IN).

PLANT EXPRESSION SYSTEMS

Plants offer several advantages compared to other recombinant protein expression systems; these include the possession of eukaryotic post-translational modification machinery, simple low-cost scale up for manufacturing, and safety of use of plant-derived products in humans or animals due to the lack of any harbored mammalian pathogens. Moreover, proteins produced in plants are free from toxins that may contaminate preparations from bacteria or mammalian cell culture. N-linked glycosylation is a post-translational modification which is important for folding of some eukaryotic proteins. Mammalian glycoproteins are efficiently glycosylated when they are expressed in transgenic plants. Although in both mammalian and plant cells the glycan processing machinery is located in the endoplasmic reticulum (high mannose) and Golgi apparatus (complex glycan), plants are unable to perfectly perform the human-type glycosylation of biopharmaceuticals. This occurs because of some structural differences between plant and mammalian N-linked glycans; namely, plant complex N-linked glycans contain β1,2-xylose and α1,3-fucose residues which are not present in complex glycans of humans. Recently, there have been many efforts towards humanization of N-linked glycosylation and N-linked glycans of biopharmaceuticals expressed in plants (Frey et al., 2009; Saint-Jore-Dupas et al., 2007; Strasser et al., 2009; Vyzina et al., 2009; Matsuo and Matsumura, 2010).

The approaches for target protein production in plants evolved rapidly and have resulted in the development of two main strategies such as transgenic and transient target expression. In the transgenic route, the target gene is incorporated into the plant nuclear genome or chloroplast genome, while in the transient route genetically engineered plant viruses are introduced into plant and the recombinant protein is expressed without prior integration into the plant host genome.

Transgenic plants

Historically, recombinant proteins in plants were produced through the introduction of a target gene into the nuclear genome (Franken et al., 1997; Daniell et al., 2001). More recently, chloroplasts have been engineered for candidate vaccine production (Tregoning et al., 2004; Koya et al., 2005; Molina et al., 2005; Daniell, 2006). The majority of target proteins expressed in plants are vaccine antigens, including enterotoxigenic *E.coli* Lt-B antigen (Haq et al., 1995), *Bacillus anthracis* protective antigen (Aziz et al., 2002), Norwalk virus capsid protein (Huang et al., 2005), and Hepatitis B surface antigen (Richter et al., 2000; Sunil Kumar et al., 2003). These plant-produced vaccine antigens generate pathogen-specific protective immune responses when administered into animals (Carrillo et al., 1998; Tuboly et al., 2000; Khandelwal et al., 2004). By using the transgenic approach, recombinant proteins are produced in both whole growing plants and plant cell cultures via conventional fermentation (Schillberg et al., 2005; Boehm, 2007; Floss et al., 2007).

In spite of the advantages such as lower production cost, possession of eukaryotic post-translational modification machinery, and target protein stability, the transgenic plant approach has some concerns which are mainly associated with

the long development time, and for nuclear transgenics, with low target accumulation levels and the possibility of gene flow from *transgenic plants to wild types.* In comparison to stable transformation, transient expression systems (see below) circumvent the above mentioned concerns and is potentially the most rapid and cost-efficient system for the production of recombinant proteins.

Plant-based transient expression

Transient expression of target proteins in plants is based on the introduction of an expression vector into plant tissue either directly as plasmid DNA or as *in vitro* synthesized RNA transcript, or indirectly via an *Agrobacterium tumefaciens*-delivered expression cassette, or "launch vector" (Musiychuk et al., 2007). Transient gene expression has a number of advantages compared with the stable transformation, such as time efficiency, high level of target protein expression, uniformity and consistency of target accumulation, scalability, and fewer environmental concerns due to contained facility production. In transient expression systems, plant RNA viruses are used as vectors for foreign protein expression (Pogue et al., 2002; Canizares et al., 2005; Grill et al., 2006; Yusibov et al., 2006; Roy et al. 2010). Availability of infectious cDNA clones, small genome size, ease of genetic manipulations, and a short time of target protein expression make this strategy particularly attractive. In addition, there is no need to genetically alter host plants. As the target gene is inserted into the viral genome, the transgene is amplified upon infection of the host plant and the recombinant protein expression is transient. Both replication of the viral vector and expression of the target gene are limited to the cell cytoplasm. To date, several plant RNA viruses have been used to develop expression vectors, including *Tobacco mosaic virus* (TMV), *Potato virus* X, *Alfalfa mosaic virus* (AlMV), and *Cowpea mosaic virus* (Pogue et al., 2002; Yusibov and Rabindran, 2004; Roy et al., 2010). There are different approaches for the expression of foreign sequences using plant viruses. The most commonly used approaches for producing soluble protein antigens are: i) replacing non-essential viral genes such as coat protein with target sequence, and ii) inserting target sequence into the viral genome as an additional gene whose expression is driven by a second coat protein subgenomic promoter. Several reviews are available on the use of this technology (Scholthof et al., 1996; Yusibov et al., 1999; Yusibov and Rabindran, 2004; Gleba et al., 2007).

Another approach which is most frequently used with plant viruses is based on fusing known target peptide epitopes to the viral coat protein to produce virus-like particles (VLPs) that present the epitopes on their surfaces. These VLPs can then be used as immunogens. Several plant virus coat proteins have been used as VLPs to produce and deliver antigenic determinants from a variety of viral and bacterial pathogens, and these particles have conferred protective immunity against the target pathogen (Pogue et al., 2002; Canizares et al., 2005; Grill et al., 2006; Yusibov et al., 2006). The peptides range in size from a few amino acids up to >150 amino acids. Advantages of the VLP strategy, include the ease of their purification from infected plant material and enhanced immunogenicity of the peptide epitopes presented on the surface of VLPs. Using AlMV, we have shown that recombinant AlMV particles presenting a 21-mer peptide from the *Respiratory syncytial virus* (RSV) G protein induced significant pathogen-specific immune responses *in vitro* in human dendritic cells and *in vivo* in non-human primates (Yusibov et al., 2005). The results showed that human dendritic cells armed with AlMV-RSV G generated vigorous CD4+ and CD8+ T cell responses, and non-human primates that received these particles responded by mounting strong cellular and humoral immune responses.

LAUNCH VECTOR SYSTEM

Both the transgenic and viral vector-based approaches for producing target proteins in plants have some shortcomings. The transgenic approach suffers from long lead times, low levels of target expression, gene silencing, and non-uniform expression. Plant virus vectors, on the other hand, provide high levels of target protein in a time-efficient manner, but the expression efficiency is determined by the capacity of the particular vector to spread throughout the plant. The latter, in turn, has been shown to cause a loss of foreign gene expression due to genetic instability of the viral vector, thus limiting the use of this technology for manufacturing purposes. To overcome the problems associated with both the transgenic and plant virus-based approaches, we and other groups have utilized the positive aspects of both systems by incorporating the plant viral vector genome into a binary plasmid of *Agrobacterium* (Gleba et al., 2005, Musiychuk et al., 2007). One of the plant viruses that we have used as a vector is TMV. In our "launch vector" system, the target gene replaces the coat protein coding sequence in the TMV genome, and then millions of copies of the "launch vector" are introduced within *Agrobacterium* into *Nicotiana benthamiana* plants by vacuum infiltration. Primary transcripts (which contain the recombinant viral genome) are then produced and transported into the cytoplasm. Subsequently, viral RNA sequences rep-

licate to very high copy numbers and high-level target protein accumulation occurs in a matter of days. Levels of target protein expression that can be achieved using the "launch vector" strategy are in the range of hundred milligram to gram quantities. The "launch vector" design circumvents instability issues related to the presence of coat protein and phloem-mediated movement, and provides additional environmental safety, because the system does not produce infectious particles.

Using the "launch vector" system, a wide variety of target antigens from several pathogens including *Bacillus anthracis*, *Yersinia pestis*, *Influenza virus*, *Plasmodium falciparum*, *Trypanosoma brucei*, and *Measles virus* have been produced in *N. benthamiana* plants at Fraunhofer USA Center for Molecular Biotechnology. For example, Domain 4 of PA from *B.anthracis* was produced as a fusion with a thermostable carrier protein derived from *Clostridium thermocellulum* (LicKM; Musiychuk et al., 2007). When combined with purified plant-produced Domain 1 of the *B.anthracis* lethal factor, which was similarly expressed as a fusion protein in plants using another TMV-based expression vector, and evaluated in mice, all animals developed high antibody titers that neutralized the effects of the lethal toxin in an *in vitro* assay (Chichester et al., 2007). In another example, the purified E7 oncoprotein from HPV that was fused with LicKM and expressed in plants using the "launch vector" system was evaluated in animals as a potential therapeutic vaccine candidate. The antigen induced target-specific IgG and cytotoxic T cell responses in mice, and conferred protection against challenge with tumor cells expressing E7 (Massa et al., 2007). Furthermore, when animals previously challenged with E7-expressing tumor cells were immunized with the plant-produced E7 vaccine candidate, all animals remained tumor-free for the duration of the 10-week study, whereas only 40% of animals immunized with *E.coli*-produced E7 were protected against tumor development. Animals immunized with the thermostable carrier protein LicKM alone developed tumors in 4 weeks. In yet another study, when F1 and LcrV of *Y.pestis* were expressed in plants, again as fusions to LicKM, purified, and administered to cynomolgus macaques, the animals developed target-specific serum IgG and IgA, and were completely protection against lethal challenge with *Y.pestis* (Mett et al., 2007). Taken together, these results demonstrate that plant-produced vaccines are effective in inducing protective immune responses not only in animals such as mice, but in non-human primate models as well.

Overall, the "launch vector" system provides high levels of expression, and is highly amenable to rapid large-scale production of a wide range of recombinant proteins and VLPs. Thus, it is a very viable platform for rapid manufacture of recombinant proteins for commercial purposes. A further advantage of the "launch vector" technology is that it lends itself to the creation of production modules with pre-determined capacity, and by simply adding more modules, manufacturing capacity can be rapidly increased.

CONCLUSION

Vaccines are vital to public health. An effective vaccine should be safe, easy to administer, stable, inexpensive, and should provide protective immunity that is sustained for long periods of time and have few side effects. There are several diseases for which vaccines are not available or current manufacturing technologies have limitations. As the industry moves forward, collaborative efforts of researchers, e.g. protein chemists and immunologists, and chemical engineers are vital to the successful development of vaccines against a wide range of diseases that can reach the global population. Currently, several novel expression systems are being explored for the production of vaccine antigens, including mushrooms, duckweed, algae and plants. The plant-based technology that we have developed (the "launch vector" system) combines the use of highly expressing plant virus-based systems and efficient delivery of target genes into plants by *Agrobacterium*. This system is highly amenable to production of a wide range of recombinant proteins and VLPs, and offers great promise for rapid, large-scale manufacturing of subunit vaccines and other therapeutic proteins. A further advantage of the "launch vector" technology is that it lends itself to the creation of production modules with pre-determined capacity, and by simply adding more modules, manufacturing capacity can be rapidly increased.

REFERENCES

Aziz M.A., Singh S., Anand K., P, Bhatnagar R. (2002) Expression of protective antigen in transgenic plants: a step towards edible vaccine against anthrax. Biochem. Biophys. Res. Commun. **299:** 345-351.

Boehm R. (2007) Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms. Ann. N.Y. Acad. Sci. **1102:** 121-134.

Canizares M.C., Lomonossoff G.P., Nicholson L. (2005) Use of viral vectors for vaccine production in plants. Immunol. Cell Biol. **83:** 263-270.

Carrillo C., Wigdorovitz A., Oliveros J.C., Zamorano P.I., Sadir A.M., Gomez N., Salinas J., Escribano J.M., Borca M.V. (1998) Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J. Virol. **72:** 1688-1690.

Chichester J.A., Musiychuk K., de la Rosa P., Horsey A., Stevenson N., Ugulava N., Rabindran S., Palmer G.A., Mett V., Yusibov V. (2007) Immunogenicity of a subunit vaccine against *Bacillus anthracis*. Vaccine **25:** 3111-3114.

Daniell H. (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol. J. **1:** 1071-1079.

Daniell H., Streatfield S.J., Wycoff K. (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Pl. Sci. **6:** 219-226.

Floss D.M., Falkenburg D., Conrad U. (2007) Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: an overview. Transgenic Res. **16:** 315-332.

Franken E., Teuschel U., Hain R. (1997) Recombinant proteins from transgenic plants. Curr. Opin. Biotechnol. **8:** 411-416.

Frey A.D., Karg S.R., Kallio P.T. (2009) Expression of rat beta(1,4)-N-acetylglucosaminyltransferase III in *Nicotiana tabacum* remodels the plant-specific N-glycosylation. Plant Biotechnol. J. **7:** 33-48.

Gasdaska J.R., Spencer D., Dickey L. (2003) Advantages of Therapeutic Protein Production in the Aquatic Plant Lemna. Bio.Processing.: 49-56.

Gleba Y., Klimyuk V., Marillonnet S. (2005) Magnifection--a new platform for expressing recombinant vaccines in plants. Vaccine **23:** 2042-2048.

Gleba Y., Klimyuk V., Marillonnet S. (2007) Viral vectors for the expression of proteins in plants. Curr. Opin. Biotechnol. **1:** 134-141.

Grill L.K., Palmer K.E., Pogue G.P. (2006) Use of Plant Viruses for Production of Plant-Derived Vaccines. Crit. Rev. Pl. Sci. **24:** 309-323.

Haq T.A, Mason S., Clements J.D., Arntzen C.J. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science **268:** 714-716.

Houdebine L.M. (2000) Transgenic animal bioreactors. Transgenic Res. **9:** 305-320.

Huang Z., Elkin G., Maloney B.J., Beuhner N., Arntzen C.J., Thanavala Y., Mason H.S. (2005) Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. Vaccine **23:** 1851-1858.

Khandelwal A., Renukaradhya G.J., Rajasekhar M., Sita G.L., Shaila M.S. (2004) Systemic and oral immunogenicity of hemagglutinin protein of rinderpest virus expressed by transgenic peanut plants in a mouse model. Virology **323:** 284-291.

Koya V., Moayeri M., Leppla S.H., Daniell H. (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect. Immun. **73:** 8266-8274.

Lowy D.R., Schiller J.T. (2006) Prophylactic human papillomavirus vaccines. J. Clin. Invest. **116:** 1167-1173.

Massa S., Franconi R., Brandi R., Muller A., Mett V., Yusibov V., Venuti A. (2007) Anti-cancer activity of plant-produced HPV16 E7 vaccine. Vaccine **25:** 3018-3021.

Matsuo K., Matsumura T. (2010) Deletion of fucose residues in plant N-glycans by repression of the GDP-mannose 4,6-dehydratase gene using virus-induced gene silencing and RNA interference. Plant Biotechnol J.: 1-18 (Epub ahead of print).

Mett V., Lyons J., Musiychuk K., Chichester J.A., Brasil T., Couch R., Sherwood R., Palmer G.A., Streatfield S.J., Yusibov V. (2007) A plant-produced plague vaccine candidate confers protection to monkeys. Vaccine **25:** 3014-3017.

Molina A., Veramendi J., Hervas-Stubbs S. (2005) Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. Virology **342:** 266-275.

Musiychuk K., Stevenson N., Bi H., Farrance C.E., Orozovic G., Brodelius M., Brodelius P., Horsey A., Ugulava N., Shamloul A.M., Mett V., Rabindran S., Streatfield S.J., Yusibov V. (2007) A launch vector for the production of vaccine antigens in plants. Influenza and other Respiratory Viruses **1:** 19-25.

Pogue G.P., Lindbo J.A., Garger S.J., Fitzmaurice W.P. (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu. Rev. Phytopathol. **40:** 45-74.

Rai M, Padh H. (2001) Expression systems for production of heterologous proteins. Curr. Sci. **80:** 1121-1128.

Rasala B.A., Muto M., Lee P.A., Jager M., Cardoso R.M., Behnke C.A., Kirk P., Hokanson C.A., Crea R., Mendez M., Mayfield S.P. (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii. Plant Biotechnol. J. **8:** 719-733.

Rogan D., Babiuk L.A. 2005. Novel vaccines from biotechnology. Rev. Sci. Tech . of Int. Epiz. **24:** 159-174.

Richter L.J., Thanavala Y., Arntzen C.J., Mason H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat. Biotechnol. **1:** 1167-1171.

Roy G., Weisburg S., Rabindran S., Yusibov V. (2010) A novel two-component Tobacco mosaic

virus-based vector system for high-level expression of multiple therapeutic proteins including a human monoclonal antibody in plants. Virology **405:** 93-99.

Saint-Jore-Dupas C., Faye L., Gomord V. (2007) From planta to pharma with glycosylation in the toolbox. Trends Biotechnol. **25:** 317-323.

Schillberg S., Twyman R.M., Fischer R. (2005) Opportunities for recombinant antigen and antibody expression in transgenic plants - technology assessment. Vaccine **23:** 1764-1769.

Scholthof H.B., Scholthof K.B., Jackson A.O. (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. Annu. Rev. Phytopathol. **34:** 299-323.

Sijmons P.C., Dekker B.M., Schrammeijer B., Verwoerd T.C., van den Elzen P.J., Hoekema A. (1990) Production of correctly processed human serum albumin in transgenic plants. Nat. Biotechnol. **8:** 217-221.

Strasser R., Castilho A., Stadlmann J., Kunert R., Quendler H., Gattinger P., Jez J., Rademacher T., Altmann F., Mach L., Steinkellner H. (2009) Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous beta1,4-galactosylated N-glycan profile. J. Biol. Chem. **284:** 20479-20485.

Sunil Kumar G.B., Ganapathi T.R., Revathi C.J., Prasad K.S., Bapat V.A. (2003) Expression of hepatitis B surface antigen in tobacco cell suspension cultures. Protein Expr. Purif. **32:** 10-17.

Tregoning J., Maliga P., Dougan G., Nixon P.J. (2004) New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC. Phytochem. **65:** 989-994.

Tuboly T., Yu W., Bailey A., Degrandis S., Du S., Erickson L., Nagy E. (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. Vaccine **18:** 2023-2028.

Vyzina L.P., Faye L., Lerouge P., D'Aoust M.A., Marquet-Blouin E., Burel C., Lavoie P.O., Bardor M., Gomord V. (2009) Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol. J. **7:** 442-455.

Yusibov V., Mett V., Mett V., Davidson C., Musiychuk K., Gilliam S., Farese A., Macvittie T., Mann D. (2005) Peptide-based candidate vaccine against respiratory syncytial virus. Vaccine **23:** 2261-2265.

Yusibov V., Rabindran S. (2004) Plant viral expression vectors: History and Developments. In: Eds. R.Fischer, S.Schillberg. Molecular Farming. Weinheim: Wiley-VCH Verlag GmbH and Co. KgaA: 77-90.

Yusibov V., Rabindran S., Commandeur U., Twyman R.M., Fischer R. (2006) The potential of plant virus vectors for vaccine production. Drugs R&D **7:** 203-217.

Yusibov V., Shivprasad S., Turpen T.H., Dawson W., Koprowski H. (1999) Plant viral vectors based on tobamoviruses. Curr. Top. Microbiol. Immunol. **240:** 81-94.