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Chapter 10

Plant-Based Peroral Vaccines

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1 HISTORY AND PRINCIPLES OF PLANT-MADE VACCINES

Peroral plant-made vaccines appeared as a part of a more wide-ranging proposition, namely obtaining of preparations of medical-biological assignment by using genetic engineering: biopharming.

At the base of a plant-based vaccine (PMV) is disposed the genetic transformation of plants with different methods, including agrobacterial transformation via *Agrobacterium tumefaciens* in which plasmids harbor genetic constructs with genes of interests and regulatory genes. Due to intrinsic native capability of *A. tumefaciens* for DNA transfer into plants, there was possible to transfer genes of interest successfully to plant organisms.

The methodology of agrobacterial transfer of recombinant T-DNA to plants rendered to be very successful, and important transgenic plants were created that produced proteins of medical destination: somatotropin, serum albumin, epidermal growth factor, α -interferon, hirudin, erythropoietin, glucocerebrosidase, α - and β -hemoglobins, cytokines IL-2, IL-4, IL-10, IL-12, IL-13, and IL-18, α 1-antitrypsin, lactoferrin, and an entire row of other compounds. This direction was rapidly developed, and about 100 therapeutic proteins are produced in transgenic plants, the amount being seriously extended (Fischer and Emans, 2000).

Stably inherited in next generations, the classic agrobacterial transformation consists of the insertion of the single copy of T-DNA into the genome of host plants, followed by integration. The stability of the integration in the structure of the plant host genome is supported by the selection in the growing of explants of the next seed generation on the nutritive media, with high content of the selective antibiotic. We obtained transgenic plants synthesizing foreign proteins up to 10th generation in our experiments, treating explants with the strong selection of the medium with high concentration of kanamycin.

The initiative for the creation of peroral vaccines on the base of transgenic plants was proclaimed in 1983 for the prophylaxis of children against hepatitis B.

This needle-free vaccination is supposed to be the reasonable alternative to vaccination via injections as, according to the World Health Organization (WHO), injection vaccination revealed 15 million cases of contamination with hepatitis B, 1 million of contamination with hepatitis C, 340,000 HIV contaminations, 3 million cases of bacterial contaminations, and 850,000 infection site abscesses (Kwon et al., 2013).

The initiative of creating peroral vaccines brought great interest, and received broad development. It was shown that transgenic plants with inserted genes of interest appeared to be the system in that many antigenic proteins from dangerous

viruses and bacterial contagious agents might be produced. During 20 years after the start of the initiative of the creation of peroral or so-called initially “edible” vaccines, about 700 PMV were developed against a range of serious diseases, as follows: enterotoxigenic *Escherichia coli*, *Vibrio cholera*, *Plasmodium falciparum*, foot-and-mouth disease, rabies virus, Norwalk virus, rotavirus, hepatitis B virus, hepatitis C virus, hepatitis A virus, measles, rubella, human immunodeficiency virus, *Staphylococcus aureus*, rhinovirus, papillomavirus, cytomegalovirus, *Pseudomonas aeruginosa*, respiratory syncytial virus, influenza virus, *Bacillus anthracis*, hemorrhagic virus, gastroenteritis virus, pneumonia pasteurellosis, parvovirus, and many others (Streatfield and Howard, 2003).

During the years 2001–07, we made an attempt to develop PMV against dangerous diseases, in particular, a binary vaccine against HIV-1 and hepatitis B (Salyaev et al., 2010, 2011; Shchelkunov et al., 2006), and another against hepatitis B (Salyaev et al., 2012). In these investigations, the authors carried out mostly fundamental characteristic research, because transgenic plants on a base of nuclear agrobacterial transformation often produced only low amounts of antigenic proteins. Nevertheless, it is known there are examples of high expression of foreign proteins in plants: phytase in tobacco (14% of total soluble protein, TSP), or eubacterial glucanase in *Arabidopsis* (26% TSP). But, as a rule, the general nuclear stable transformation provided the production of foreign genes of interest in transgenic plants at a level of 0.06%–0.1% TSP, as follows: B subunit of thermolabile enterotoxin of *E. coli* LTB (0.01% TSP), HBsAg HBV (0.01% TSP), glycoprotein S coronavirus of transmissible gastroenteritis (0.06% TSP), human serum albumin (0.02% TSP), human complement C protein (0.001% TSP), epidermal growth factor (0.0001% TSP), erythropoietin (0.026% TSP), β -interferon (0.000017% of fresh weight) (from Streatfield and Howard, 2003).

But, regardless of the low production of target proteins, almost all produced PMV and foreign proteins were immunoreactive, capable to activate the immune response both in experimental animals and volunteers. The most significant fact in these experiments was the appearance of immunogenic properties, as well as the preventive disposition of antigenic proteins synthesized in transgenic plants at the concentration ranging from 0.00001% to 0.000001% fresh weight (Streatfield and Howard, 2003). Later, it was shown that PMV are capable to induce both mucosal and general systemic immune response in mammals. Due to many positive characteristics, PMV are needed to be switched to commercial levels.

However, as mentioned previously, the standard agrobacterial transformation had low productivity, as a rule, owing to the insertion of a single copy of T-DNA, and that has not facilitated the high production of the synthesis of antigenic protein per 1 unit of plant mass. Moreover, because of using virus genes in the expression constructs, and its integration into the genome of the host plant, there appeared epigenetic disorders that resulted in chimerization and in silencing. This phenomenon is well documented at the present time, and is called RNA interference (RNAi), leading to virus-induced-gene-silencing (VIGS); this functions in nature as a defensive antiviral mechanism.

In 1987, at Stanford University, J. Sanford proposed the idea of a fast and effective plant transformation by using the process of bioballistics, in which the DNA of target genes or recombinant plasmids were placed upon tungsten or gold microprojectiles placed onto the surface of teflon macroprojectiles. Both were then shot onto plant objects. This method received significant development because not only plants might be transformed in this way, but also other study objects, such as mice, frogs, rats, and their organs. The method was named as “biolistic.” Subsequently, this method and “gene guns” of different design, working at the base of inert gases (helium, argon), occupied the main position in the biotechnology of transgenic plants, because this method allowed to shoot large recombinant plasmids with high expressive constructs (Klein et al., 1987).

The production of synthesized antigenic proteins increased up to 10–100 times. The method was merely elaborated by H. Daniell with coworkers (Chan and Daniell, 2015). They created transgenic homoplasmic–transplastomic plants with not only high antigenic production, but also that of other proteins for enzyme replacement therapy that is very useful in humans. The most productive construct made by Daniell and coworkers was the creation of polycistronic joining of target gene, marker gene, and flanking recombinant fragments of genes in the intergenic region from the plastid of host plants. The placement of the target gene into the cistronic sequence of plastid genome allowed to increase the yield of the target gene of crystal toxic protein (Cry2Aa2) of *Bacillus thuringiensis* to 45.3% TSP and even more in tobacco leaves (De Cosa et al., 2001).

Table 10.1 shows that the expression in plastid constructions exceeded up to 1000 times or more the expression at nuclear transformation. It should be mentioned that plastid transformation, with the use of selective marker genes of kanamycin resistance NPTII, resulted in the increase of the selective enzyme up to 23% TSP. Such vaccine preparations have doubtful value on account of their very high content of enzymes resistant to antibiotics.

Complex systems of plastid transformation were developed by P. Maliga who combined for the first time the agrobacterial and plastid transformation, placing the construct to a nuclear expression vector, and the gene of the target under the leader of a large subunit RUBISCO, for the transportation of gene/proteins of interest into plastid. In this way it was possible to increase the production of tetanus toxin TetC placed under promoter Pprn with regulatory elements T7G10, atpB/TrbcL up to 10%–25% TSP (Maliga, 2003).

TABLE 10.1 The Comparison of Nuclear and Plastid Expression of Target Proteins

Protein/Vaccine/Gene	Insert Site	Promoter	5'/3' Regulatory Elements	Plastid Expression, % of TSP ^a	Nuclear Expression, % of TSP ^b
Human somatotropin HST	trnV/rps12/7	Prrn, PpsbA	T7G10 or psbA/Trps16	7 or 1%	<0.001%
Human serum albumin HAS	trnI/trnA	Prrn, PpsbA	ggagg ur, PsbA/TpsbA	0.02%, 11.1% TSP	0.002%
Cholera toxin CTB	trnI/trnA	Prrn	ggagg/TpsbA	4% TSP	0.01% in leaves, 0.3% in tubers
α -Interferon IFN α 2b	trnI/trnA	Pprn	PpsbA/TpsbA	19% TSP	β -Interferon 0.000017% of TSP
γ -Interferon IFN-g	rbcL/accD	PpsbA	PpsbA/TpsbA	6% TSP	
Insulin growth factor IGF-1	trnI/trnA	Pprn	PpsbA/TpsbA	32% TSP	<0.001%

^aChan and Daniell (2015).
^bStreatfield and Howard (2003).

Nevertheless, plastid transformation is still a very laborious and time-consuming method that needs significant financial support. However, the principle of combining the methods of transformation occurred as a very useful perspective.

The use of constructs with plant virus genes and regulatory elements has been very attractive for a long time, because of its high potential in the productivity of synthesis of heterologous proteins. It is possible to add that the incorporation of regulatory elements of plant viruses into expression vectors for plant transformation increases very efficiently the production of target protein in transgenic plants.

The most negative feature of virus-based constructs is their instability. In order to save virus-based vectors from unrelated changes coming from environmental stress, Yu, Gleba and coworkers elaborated the combination of agrobacterial vector with “destructive” parts of the TMV genome in one plant virus expression vector. The method was named “magnification” because the inserted RNA-dependent RNA polymerase (RdRP) provided the increasing synthesis of RNA replicons (RNA amplimers), serving as templates for the synthesis of the target protein; in this case, the accumulation of target proteins increased up to 80% of TSP (Marillonnet et al., 2004)

Thus, developments in parallel methods of plastid and nuclear transformation were joined and, as a result, very high productive methods of plant transformation were obtained that are suitable for the demands of the commercial production of industrial proteins, vaccines, antibodies, and therapeutic biomolecules (Komarova et al., 2010; Marillonnet et al., 2004).

The different examples of genetic transformation of plants were listed in the paper by Chan and Daniell (2015).

The employment of edible plant parts as a vaccine material—whose toxicity is practically close to zero due to the absence of cryptic pathogens that are potentially dangerous for humans, prions, harmful plant viruses, and other microbial pathogens—is very advisable. There is a point of view that, during transgenesis and recombination in plants, some features that can elicit allergy might be potentially revealed, as well as eliciting different types of autoimmune diseases, cardiovascular collapse, and disorders of central nervous system. However, from this point of view, the other expression systems (bacterial, baculoviral, yeast) have to appear to have more chances to realize cryptic dangerous properties as a result of recombination; for example, the presence of silenced transposons and retrotransposons that are capable to evoke serious diseases of epigenetic character. Therefore, even from this sophisticated position, plants as expression systems are safer and significantly more reliable candidates for the production of recombinant proteins, as well as peroral vaccines.

In Fig. 10.1, examples of different expression systems are presented. These are employed at the present time in biopharming for the production of target proteins of different destinations, and to diverse extents. Plant expression systems are best, according to the opinion of many authors (Peyret and Lomonosoff, 2015), owing to their safety, low cost, easier shifting to scalability, as well as having more appropriate folding, glycosylation, and other processes of posttranslational modifications of the proteins synthesized.

1.1 Plant Expression Systems Based on Plant Virus Constructs

As was mentioned before, investigations on employing “destructive” plant viruses (both RNA viruses and DNA viruses, equally) have attracted significant interest over the past 10 years. The very active virus enzyme replicase or RNA-dependent RNA polymerase is used in these works, in which the sequence is placed in frame with the gene of interest in genetic constructs. The promoter of the cauliflower mosaic virus (CaMV) p35S that is capable to transcribe the polycistronic sequences

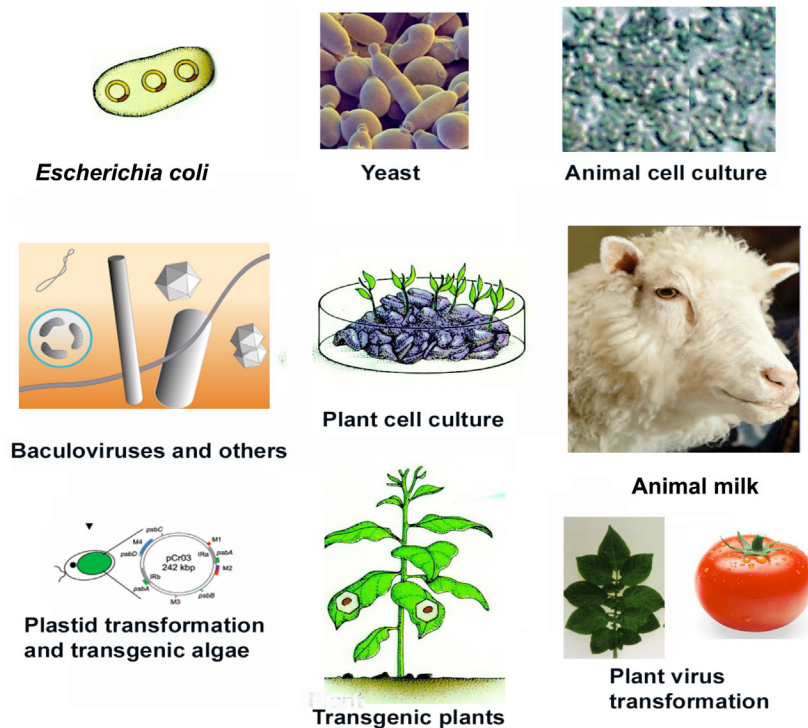


FIGURE 10.1 Expression systems for the production of recombinant proteins.

of genes is employed; p35S also facilitated the episomal expression of foreign genes in plants similar to bacterial plasmids or DNA viruses in mammals, such as SV40 (Peyret and Lomonossoff, 2015).

The construct is placed in the space between RB and LB of T-DNA for the control of virus segments, and for the stabilization of the processes of the transformation. T-DNA is disarmed in this process and is free from its own plant oncogenes.

If virus genes encoded the synthesis of the moving protein, or if the capsid proteins are placed in the construct, the pseudovirions formed will extend throughout the plant.

A replicase RdRP after the synthesis of the first primary transcript that occurs as supposed in the nucleus moves to cytoplasm, as a ready and very effective enzyme, and initiates the processes of the replication of sequences followed downstream (Fig. 10.2).

Large amounts of amplicons are formed and because of this p35S joins the circles and, therefore, there are formidable amounts of replisomes that appear and serve as templates for the synthesis of antigenic proteins, as well as other target proteins or biomolecules. The usage of the plant translational machinery is so rapid and effective that, continuing at such a rate, around several micrograms of target protein per 1 g of fresh weight are accumulated in plant tissues after 3–4 days from agrobacterial infection. However, the infected plant usually dies after 7–10 days.

The appearance of virus sequences in plant cell evokes usually the phenomenon of RNAi and, in order to escape the RNAi, the virus genome sequence encodes any virus protein-antisilencer that might be placed into genetic construct. This placement will essentially increase the production of the target antigenic protein or other protein synthesized in this system.

Thus, the use of plant virus expression vectors significantly promotes the sharp increase of the amount of antigenic proteins obtained for the vaccine material. Here, the amount of antigenic proteins exceeds the amount of antigenic proteins obtained by the biolistic method.

2 GLYCOSYLATION OF ANTIGENIC PROTEINS

Vaccine antigenic proteins created in plant virus expression systems are considered to be glycosylated. Significant amounts of sites for glycosylation on the surface of antigenic proteins derive naturally from many sites of glycosylation of virus capsid proteins or other gp of viruses. This shows the real evidence for overall glycosylation.

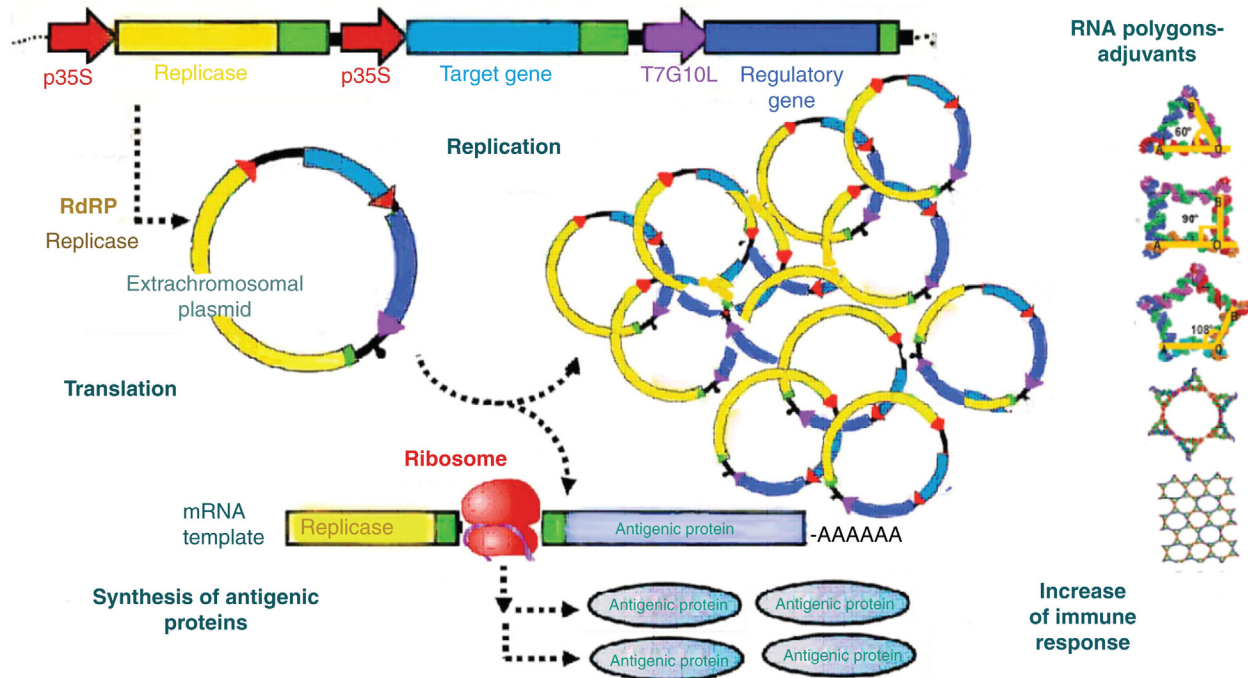


FIGURE 10.2 Scheme of the synthesis of replicons by using of RdRP and further assembling of RNA replicons into replisomes and RNA polygons.

As many as about 170 families of plants glycosyltransferases were found, and they function in the binding of xenobiotics, in order to withdraw them in bound state to the vacuole space or for transport with the aim of secretion, thereby detoxifying them. Unconditionally, antigenic virus proteins of dangerous virus pathogens, as much plant transgenic practice shows, are not very friendly proteins for plant metabolism. However, it is possible for distinct limits of plant cell to survive, but only if antigenic virus proteins might bind in fast synthesized conjugates with the activated glucose as the most abundant source in the plant cell set of UDP-transferases or with other glucoside residues.

2.1 *ugt* Gene Encoding UDPG-Transferase From *Zea mays* L.

2.1.1 Usage in the Creation of High Productive Transgenic Plants With Enriched Content of UDPG-Transferases and Development of PMV

The gene *ugt* was isolated from green plantlets of *Zea mays* L. by the method of screening positive clones from the cDNA library Lambda ZAP II (Stratagene, USA) of corn with antibodies grown in rabbit serum to IAA-synthase (UDPG-transferase) in 1993, at Michigan State University. This was done in the laboratory headed by professor R.S. Bandurski, with our participation (Rekoslavskaya et al., 2002).

2.1.2 The Characteristic of the Gene *ugt*

According to Professor R.S. Bandurski (Szerszen et al., 1994), UDPG-transferases play a significant role in the growth and development of plants, in plant hormone metabolism, in secondary compounds, and at the binding of lipids, phenols, peptides, and proteins (Percival and Bandurski, 1976).

The general scheme of the participation of UDPG-transferase—encoded by the gene *ugt* (IAA-glucose synthase), in different processes in plants in which the glycosylation of antigenic proteins (marked with one star) and the increase of immune response after vaccination (marked with two stars) are of the most challenging interest for the design and development of PMV (Fig. 10.3).

Beginning from our experience in obtaining transgenic plants with the *ugt* gene, the specific activity of UDPG-transferase increased significantly. According to our data, the activity of UDPG-transferase increased from 47 to 187 nmol/mg of protein per hour in tomato (Rekoslavskaya et al., 2002) and from 260 to 1670 nmol/mg of protein per hour in potato (Salyaev et al., 2004).

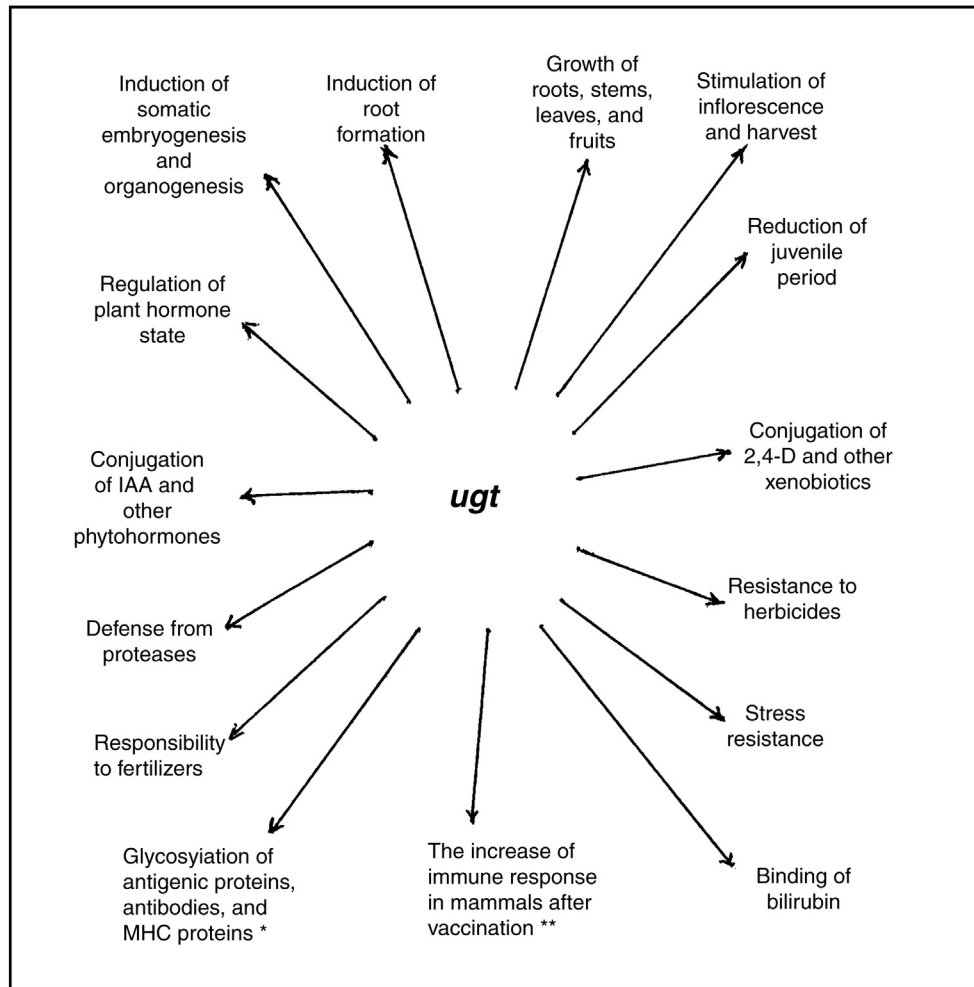


FIGURE 10.3 Scheme of multiple participation of the gene *ugt*, encoded the synthesis of UDPG-transferase in different processes.

Consequently, the significant part of proteins in transgenic plants undergoes the process of glycosylation, but in this circumstance, as we can see from further consideration of the connection of the glycosylation and the processes of vaccination, it has the most important value because it essentially mediates downstream functions.

2.2 Usage of *ugt* Gene as Selective Gene in the Design and Development of PMV

As mentioned previously, transgenic plants can express significant amounts of selective proteins that give resistance to antibiotics up to 23% of the TSP that is undesirable.

Therefore, we elaborated the method of selection of regenerants of tomato transgenic plants on the medium of Murasige and Skoog (MS), containing 0.7 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). For this selection the gene *ugt* was inserted into the genetic construct instead of *nptII*.

The capability of root formation of explants of tomato transgenic plants with the gene *ugt* and in the control on the MS medium with 0.7 mg/L 2,4-D is shown in Fig. 10.4. Control explants were not able to form roots on MS medium with 2,4-D but the transgenic explants were rooted normally, and then selected transgenic plants grew successfully and gave a good harvest (Fig. 10.5).

2.3 Immunogenicity and Other Advantages of PMV

We used tomato fruits for transformation with the genetic construct with HPV16 L1 (human papillomavirus type 16 major capsid protein L) in our work because obtaining of adequate plants from regenerants with the gene *HPV16* L1 had



FIGURE 10.4 Selection of explants of transgenic with gene *ugt* tomato on Murashige and Scoog medium with 0.7 mg/L 2,4-D.



FIGURE 10.5 Transgenic tomato with the gene *ugt* growing from explants in greenhouse after selection on 0.7 mg/L 2,4-D.

failed—after the transfer from in vitro into the soil of the greenhouse, the selected regenerants died very soon. This was seen in the work of [De la Rosa et al. \(2009\)](#), in which only 1%–3% transgenic plants from large amount of regenerants with the gene *HPV16 L1* had survived; as such, selected plants had very reduced generative development and formed 2–3 small fruits only. Therefore, we used transformation directly into the fruits by the injection of agrobacterial suspension with gene *HPV16 L1* into the basal part of fruit.

[Hongli et al. \(2013\)](#) made the transformation of tobacco leaf disks with *HPV16 L1* and obtained selected regenerants. Grown regenerants were extracted with the buffer, the extract was freeze-dried and the dry material was dialyzed. The preparation obtained was used as a vaccine in order to study immunogenicity on mice. The antibody response was weak when mice were vaccinated four times with a dose of 0.38 mkg, and the ELISA readings were lower than 1 unit of optical density after the measurement. In order to improve the immune response, mice were given thermolabile nontoxic LTB subunit B of enteropathogenic *E. coli*. The immune response increased greatly with the combined administration of *HPV16 L1* vaccine material and LTB in mice serum. However, it was found that the use of LTB during peroral vaccination resulted in the destruction of gastrointestinal tissues of mice, revealing bloodying and swelling.

Since tomato fruits contained a great number of natural adjuvants (tomatine, lycopersicine, esculetin, lycopene, tomatidines), there was no necessity in our work to add any artificial adjuvants for the induction or activation of immune response

in mice during vaccination. Moreover, as in the work by [De la Rosa et al. \(2009\)](#), the intact freeze-dried ground material of tomato fruits, without any isolation and purification of antigenic proteins, was used for mice vaccination in our work. Our group found that any manipulations for isolation, purification, and concentration in vacuum, concentrator of antigenic protein HPV16 L1, as well as SDS electrophoresis resulted in instability and in the loss of immunogenicity. The immunogenic capability of antigenic protein HPV16 L1 depends on the correct natural orientation of linear and conformational epitopes of the quaternary protein structure. Chemical or electrostatic forces held the natural conformation of epitopes on the virus capsid, and are strong enough to resist. But given the impact was in excess of the strength of binding between the amino acids of antigenic protein, for example, heating for 10 min at 100°C or 30 s in the microwave oven (our unpublished data), as well as using salt denatured buffer systems, immunogenicity (binding with antibodies) or in vivo with effector molecules of complement—as well as with mucosal immune structures of gut epithelial cells lining (GALT) mucosal associated lymphoid tissues (MALT) and other lymphoid tissues—was destroyed. Perhaps that was the reason for use of vaccine material in very high doses of virus-like particles (VLP) with large quantities of 40 mkg of each antigenic proteins during vaccination with well-known commercial vaccines, such as “Gardasil” and “Cervarix” where pseudovirions were sorbed on the amorphous hydroxyphosphate sulfate aluminum that had adjuvant properties. These very hard conditions of vaccination with “Gardasil” can explain the appearance of an entire row of side effects (the statistics of vaccination are known: 1 death case per 200 vaccinated individuals).

During the usage of high doses of vaccines, a new interesting behavior of antigenic proteins HPV L1 appeared, that in high concentration were able to induce the cross-reactive interaction of antibodies of types 16, 18, 31, 33, 45, 53, 54 ([Bissett et al., 2014](#)). Previously, during standard vaccination with vaccines obtained in bacterial or in baculoviral systems, the phenomenon of cross-reactive interaction of antibodies with antigens was unknown, and actually the reverse was observed, induced immune response by one type of HPV was not suitable for any other type of HPV immunity because antibodies revealed strong specificity only to one particular type of HPV. The most sparing and commercially suitable method of the concentration of antigenic protein HPV16 L1 was freeze-drying and even here some little loss of the immunogenicity occurred. Positive results in immune response were received after drying raw vaccine material in a stream of warm air, as the readings in ELISA were given the high titer (about 3 logs) in blood serum of mice after the vaccination with air-dried vaccine material from tomato fruits with HPV16 L1 (our unpublished data).

2.4 Mucosal Vaccines. The Advantages of Peroral Vaccines

The immunogenicity of antigenic proteins is the main condition for the success of vaccines. Therefore, there are several means and techniques available in order to increase that immunogenicity ([Hooper, 2009](#); [Wolfert and Boons, 2013](#)).

Major histocompatibility complex (MHC) class I and II reside in glycoproteins that can present antigenic peptides with epitopes on the cell surface, for the recognition and binding to circulating T lymphocytes ([Wolfert and Boons, 2013](#)). Therefore, it is important to consider the modification of antigenic protein by glycans for the uptake, proteolytic cleavage, and presentation by MHC, followed by activation and priming of T cells, thus the glycosylation of antigenic proteins is very important for vaccine development. All key proteins are involved both in the recognition of antigen and in following orchestration, meaning that the harmonic interaction of effector functions in combination are glycosylated ([Wolfert and Boons, 2013](#)). Therefore, glycosylation is of great importance for the reactions involved in immune response.

It was known ([Peyret and Lomonosoff, 2015](#)) that the glycosylation of proteins descendent in plant cells was closely approximate for the immune response of mammals. Bacteria do not have similar glycosylation; the yeast type of glycosylation is very different from those of mammals and humans. The baculoviral expression system has a very distant type of glycosylation and it satisfies weakly the glycosylation of proteins in mammals.

Antigenic glycoproteins in immune tissues of mammals form spatial nanostructures (lattice type) as dendrimers that are likely to be attractive nanostructures for T and B lymphocytes, dendritic cells (DC), and for other components of cell-mediated immunogenicity. The existence of dendrimeric nanostructures of glycans of antigenic proteins allows to pass into the immunogenic zone of undermucosal layer named *lamina propria* that has no T and B lymphocytes and no DC, but as a whole this zone plays a very important role in immunogenicity, though previously it was supposed that *lamina propria* did not have evident structural immunogenic formations similar to lymphoid tissues (M cells in Peyer aggregated lymphatic follicles called patches).

Thus, glycoproteins of dendritic immunogenic structures of antigens are considered to be the most transportable, soluble, stable, and more effective mobile nanostructures involved in the immune response, in comparison to cell structures in M cells of Peyer patches.

The glycosylation of antigenic virus proteins corresponds to the defensive factor for virus particles, allowing them to escape the immune system of host cells masking receptors. For example, the capsid protein of HIV-1 has 25 sites for the

binding of glycosides residues. The glycosylation of capsid proteins allows making a departure from proteases and from the antibodies of first barrier defense of host cells.

The key function in the recognition of foreign antigenic proteins resides in DC that due to their functional structures, receptor domains, and metabolism form adaptive reactions of immune systems in mammals.

At the beginning of 21st century, this recognition was considered as right, and specific only for HIV virions made by DC. But by means of newly found knowledge, the representation of the role of DC in the initiation of immune response as an adaptive answer to pathogens was significantly extended.

DC are those structures that circulate in the extracellular space of tissues, finding and binding polysaccharides, polyglycans, and lipopolysaccharide molecules on the surface of invading pathogens: bacteria, viruses, microfungi. For this purpose, DC have glycosylated receptor sites that are able to form diverse polyglucoside bonds with polysaccharides and polyglycan proteins, resulting in the formation of pentamers or more complicated polyglycan dendrimeric structures, such as a lattice that gives to these dendrimeric nanostructures its distinct stability and transportability, as well as safety from the anticipatory action of proteases. The further fate of these nanostructures is well known after some events: internalization, entering lysosomes, partial cleavage by proteases, and presenting epitopes on the cell surface for recognition by receptors of T and B lymphocytes, inducing them in such way for the synthesis of specific antibodies (Wolfert and Boons, 2013).

DC are able to interact with polyglycan structures of endobiotics in the human glycome, and transfer them to the reaction with immune cells.

This question arises: how can DC choose the protein of pathogens instead of hosting its own proteins?

The key role in this recognition is played by the density of glycosylation of proteins. By this, heavy glycosylation takes place on the surface of antigenic protein, so there are more possibilities for them to be captured and be bound by DC (Wolfert and Boons, 2013).

Glycosylation plays the key role in addressing the proteins synthesized on the ER. During the so-called cotranslational glycosylation, the core oligosaccharide composed from two residues of *N*-acetylglucosamine, nine residues of mannose, and three glucose residues joins the N of Asp from the N site of newly synthesized proteins (Lehninger et al., 1993). This oligosaccharide is formed on the phosphate of donor molecule dolichol phosphate (isoprenoid derivative) docked in membranes of ER. All N-linked oligosaccharides retain a pentasaccharide core derived from the original 14 residues of oligosaccharide, and move with protein to the Golgi complex. The transferase involved in glycosylation with UDP-*N*-acetylglucosamine, UDP-mannose, and UDPG as cofactors is located on the internal luminal surface of the ER, and does not catalyze glycosylation of cytosolic proteins. At this time, the sorting of both ER and cytosolic resident proteins takes place. It is difficult to say how specific transferases to glycosyl residue bind with UDP, GDP, or ADP but, according to our unpublished data, the transferase from *Zea mays* L. encoded by the *ugt* gene uses equally both UDPG and ADPG in transgenic potato plants.

It was found that DC were responsible for the specificity of the antigen presentation (APC presentation), and for driving primary immune response, that expressed the receptor of mannose on their surface. This fact pointed directly to the interaction of glycosylate antigenic proteins made in the plant expression virus system with immunogenic DC.

The high extent of protein glycosylation in mammals provides about 200 transferases, and glycosidases per 1 cell. Moreover, approximately 7000 high reactive pentameric structures of glycosides with high capability to bind with effector molecules of complement, antibodies, and others, were found in mammalian cells. Therefore, DC chose the most heavily glycosylated proteins and surfaces of cells in the intracellular mucosal spaces when viruses with high glycosylated capsid proteins, bacteria, and microfungi with their surface polysaccharides and lipopolysaccharides enter the mucosal tissues lining MALT, GALT, anogenital tissues, tears channels (TALT), conjunctive tissues (CALT), nasal-pharyngeal tissues (NALT), and other.

Thus, DC provide sorting and the choice of cells, proteins, and other structures for the delivery to immune cells, and for the induction of the immune response.

3 PAPILLOMATOSIS, VACCINES (GARDASIL, CERVARIX), AND OUR APPROACHES TO THE PROPOSITION

Skin and anogenital warts were known even in the times of ancient Rome and Greece. It was assumed that anogenital warts were the consequence of a lascivious way of life, and the interchange of sexual partners. In the Middle Ages, according to the analysis of church books, there was found that virgins, nuns, and widows who were of honest behavior never had papillomatosis and cervical cancer.

During the years 1974–84, the cause-and-effect relation was established between cervical cancer and papillomaviruses, and in 1991–94 the vaccine against human papillomaviruses (HPV) was created.

The depressed immune system encourages the extension of cancerogenesis due to HPV infection, and therefore papillomavirus infection often combines with HIV-infection, tuberculosis, hepatitis B, hard processed types of herpes, and other dangerous diseases. All of these facts evidence the possible pandemic status of high oncogenic types of HPV associated with other dangerous viruses, prompting the problem of the actuality of the creation of vaccines of a new generation.

3.1 Preventive Vaccines

The most oncogenic types of papillomaviruses (HPV16 and HPV18) are the main pathogenic agents of the dangerous disease of cervical cancer, of which around 650,000 women die annually. There are numerous attempts conducted to create both preventive and therapeutic vaccines against papillomaviruses, in numerous laboratories in the world, and although two preventive injection vaccines—"Gardasil" (Merck) and "Cervarix" (GlaxoSmithKline)—are set up, the expansion of papillomaviruses is still in progress.

It was known that the company "Merck" (USA) developed and applied for the US Food and Drug Administration authorization of quadrivalent injection vaccine in more than 60 countries in the world. As a result of mass vaccination, the statistics reveal 50 deaths per 1000 vaccinated women, meaning one woman or girl dies after the vaccination of 200 women. That is an alarming enough indicator in the vaccination with "Gardasil." Perhaps this fact can be explained by the presence of hydroxyphosphate sulfate aluminum as adjuvant, because aluminum salts are toxic in humans.

Another injection vaccine, namely "Cervarix" (GlaxoSmithKline), is working on the base of the baculoviral expression system in insect cells of the fall armyworm *Spodoptera frugiperda* (Lepidoptera). In the expression system in the insect cells of the fall armyworm, the folding of the major capsid protein L1 occurred, but it was quite different from the folding of the native HPV L1 in capsid. Therefore, antigenic proteins produced in the baculoviral expression system might induce the synthesis of antibodies that would not be reliable for the recognition of native three-dimensional structures of capsid proteins L1 in virions of HPV. Perhaps that was the reason (as in the case of "Gardasil") for the use in administration of very high doses (up to 40 mkg) of antigenic proteins L1, together with adjuvant 3-O-deacyl-4-monophosphoryl lipid A.

The endurance of these vaccines, according to the data in scientific literature, is of 5 years.

In connection with the expansion of papillomavirus infections around the world, biotypes of HPV, the structures of genes and its regulation are actively studied, as well as the fundamental items of the interaction of HPV genes during the initiation of cancerogenesis.

In the past years, the study of the structure and the regulation of gene expression in the process of the clearing of virions reached a real progress; this occurred approximately 2 years after the first infection with papillomavirus in humans with a normal immune system, and without a following reinfection. The expansion of cervical intraepithelial neoplasia (CIN) has not taken the place. However, in some cases, the integration of oncogenic genes *HPV E6* and *HPV E7* took place and, as a result, the events connected to neoplasia increased. The early gene E2 and the early protein E2 were shown to control the integration of oncogenes, and they can influence the principle of regulatory action in the initiation of cancerogenesis (Fig. 10.6).

The increase in the amount of capsid proteins facilitates an increment in the self-assembling of virion particles, the transfer through the Golgi net, and budding from mammalian cells via exocytosis as a full size virus. This process appeared to be the final step in the clearing of virus particles. Thus, the interaction of E2 and L1 proteins can result in the liberation of cells from HPV virions.

There was evidence that capsid proteins L1 and L2 invoked the reduction of the amount of E2 protein by feedback, thus decreasing its synthesis.

From all the above, it follows that the conduct of fundamental work on the study of intrinsic aspects of neoplasia initiation is of very important significance, because there is a real dangerous expansion (even pandemic) of papillomaviruses diseases in the world.

In countries where the monitoring of papillomavirus infection between people is carried out, for example, in Italy, the annual detriment to the economy from diseases associated with papillomaviruses was counted, and constituted 680 million Euro (Baio et al., 2012). Even 5 years ago, the WHO made a prognosis on the expansion of HPV infection in women as reaching 50% until the year 2020. But now the expansion of HPV is growing, reaching around 100% of infected women.

The existing preventive vaccines give a significant effect, but they are not free from negative side features. Traditional preventive vaccines are not able to provide a 100% defense from papillomaviruses, and also have side effects. It follows that it is necessary to continue the development of vaccines of a new, innovative generation, with high effectivity and safety. In particular, vaccines based on transgenic plants, the so-called PMVs, already satisfy this demand because of their numerous positive characteristics that were demonstrated many times. In the development of such vaccines, the necessity to increase the content of antigenic protein is a matter of the greatest importance.

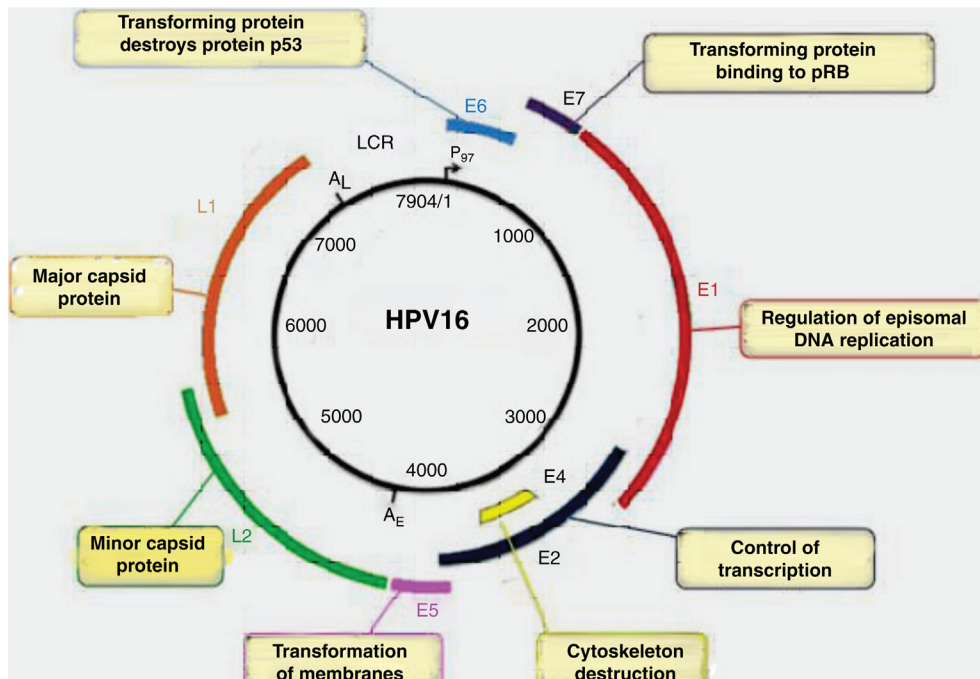


FIGURE 10.6 Physical map of papillomavirus HPV16 with indication of functions of proteins. *E*, early expression proteins; *L*, late expression proteins.

As such, we made an attempt to develop a variant of plant expression system for the synthesis of antigenic protein of high oncogenic risk HPV16 L1, under the control of promoter p35S CaMV, and the sequence of the omega leader of tobacco mosaic virus (the translational enhancer) Ω 5'UTR TMV (Salyaev et al., 2016). The usage of Ω 5'UTR TMV in the genetic construct resulted in the significant increase of the production of antigenic protein HPV16 L1 in tomato fruits infected with the suspension of *A. tumefaciens* EHA105 with pBINHPV16 L1 Ω 5'UTR TMV.

The sequence Ω 5'UTR TMV is compiled from 68 base pairs: GUAUUUUUACAACAACAAUACCAACAACAACAAA CAACAAACAACAUA CAAUUACUAUUUACAACAAUACA (Shirokikh et al., 2010). Omega leader of TMV (Ω 5'UTR TMV) is a highly-efficient enhancer of translation due to the unicity of its structure, having consecutive features: (1) the central core part consists of 10 repeats of cytidylic and adenylic residues CAA, compiled about a half of the whole sequence of the omega leader, (2) repeated CAA residues form the stable triple helix that is very different from the classical canonic Watson and Crick pairing; there are no any guanilic residues, but instead there are C:A and A:A connected with one hydrogen bond, (3) leader sequence by triple helix can bind to 80S and 40S ribosomes, and enhance the translation. Ω 5'UTR TMV is able to increase the translation from homologous or heterologous RNA in cells of different types (Shirokikh et al., 2010).

In our genetic construct, the target gene *hpv16* L1 was placed under the promoter p35S CaMV (Fig. 10.7). The sequence of Ω 5'UTR TMV put in the 5' untranslated region served as enhancer of translation. The terminator *tocs* of the gene *ocs* encoded octopine synthase from agrobacterial T-DNA was used in the construct. A poly(A) sign was present in the construct at the 3' untranslated region.

The synthesis of genetic construct was carried out by the company Genscript (USA).

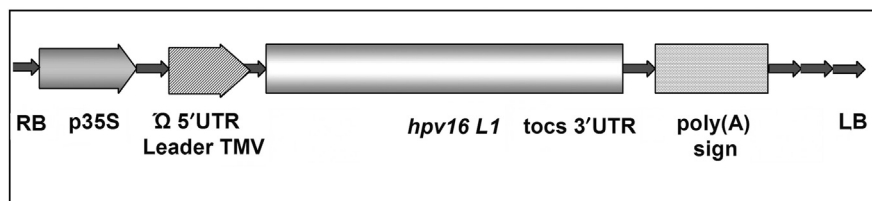


FIGURE 10.7 Genetic construct pBINHPV16 L1 Ω 5'UTR TMV designed for genetic transformation of tomato fruits.

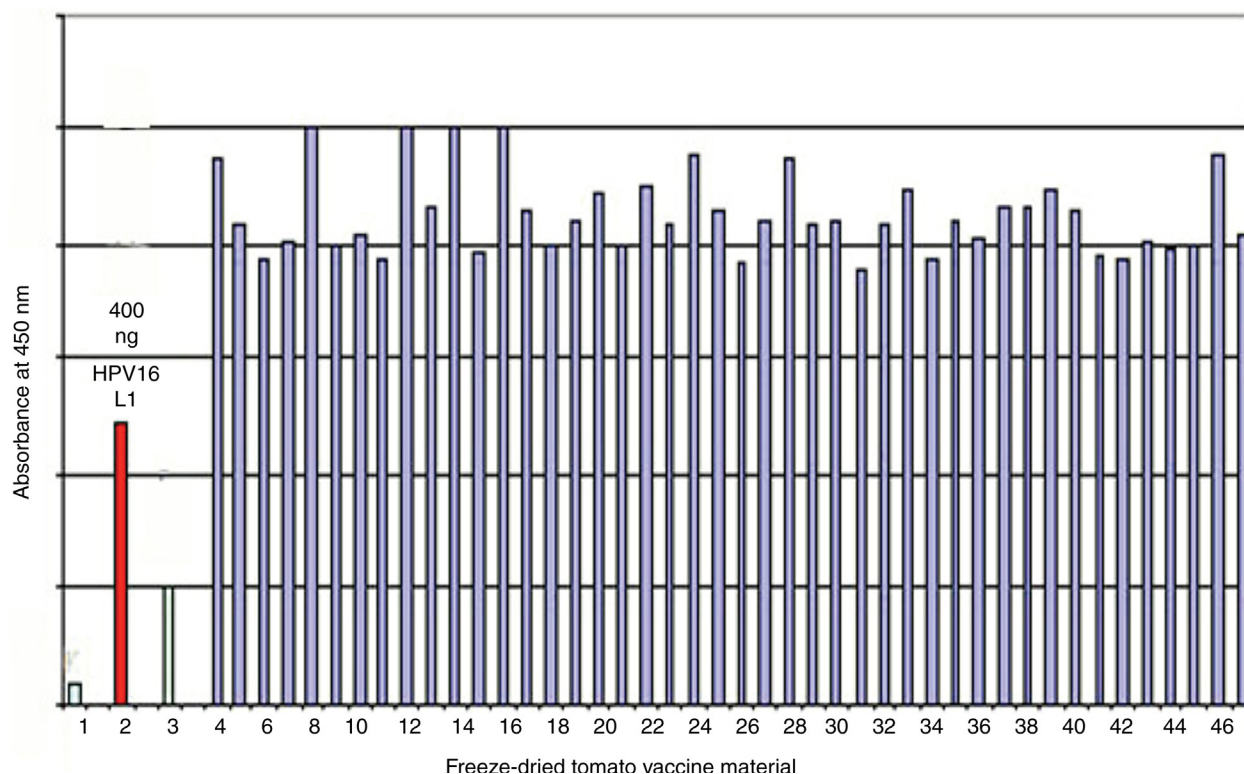


FIGURE 10.8 ELISA analyses of the production of HPV16 L1 Ω 5'UTR TMV in tomato fruits of hybrid “Pink Flamingo.” 1, Negative control; 2, standard 400 ng of HPV16 L1 (Genway Biotechnology, US); 3, nontransformed fruits; 4–5, samples of freeze-dried vaccine material from individual tomato fruits.

The genetic transformation of tomato fruits was conducted by the injection method, with the suspension of *A. tumefaciens* EHA105 with pBINHPV16 L1 Ω 5'UTR TMV directly. The maximal result of 2330 ng/mg of TSP was reached in the hybrid tomato fruits, namely the so-called “Pink Flamingo” (Fig. 10.8).

We have to mention that in our previous work without omega leader, the content of antigenic protein in tomato fruits was 50–70 ng/mg of TSP in the case of TBI-HBS antigen, and 130–211 ng/mg of TSP in the case of PreS2-S antigen.

Thus, the usage of the sequence Ω 5'UTR TMV as an enhancer of translation significantly increased the production of antigenic protein HPV16 L1, in comparison to earlier constructs used without Ω 5'UTR TMV that gave us the possibility of obtaining of vaccine material for preclinic trial of the created candidate vaccine.

3.2 Pan-Vaccine Based on Minor Capsid Protein HPV16 L2

In 2015, a new interest direction appeared in the creation of a pan-vaccine with induced broadly neutralized antibodies, developed on the basis of minor capsid protein HPV16 L2. It was known since the earlier works of the 1990s that the role of minor capsid protein HPV16 L2 was insignificant, involving keeping the major capsid protein HPV16 L1, in the lattice of capsid HPV, in the state of capsid pentamer, and holding the loop bands of HPV L1 in the right natural position, with saving of critical epitopes.

The minor capsid protein L2 as a difference to the major capsid protein L1 never revealed the strong specificity to types of HPV. The HPV16 L2 molecule is situated inside the L1 protein, and it is shielded here. Later on, it became clear that the minor protein L2 played a critical role in saving the HPV virion from first immune response, and that it did not allow the interaction with immune receptors of host mammal cells. On the basis of this phenomenon, there lies the ability of the minor L2 protein to expose temporary its own epitopes after furin cleavage of L1 pentamer, during the first steps of infection that are not reacted with the cell receptor; it is not recognized by these receptors and, as a whole event block, there followed the initiation of defensive processes. The antigenic variability of the L2 protein is low. During analysis with cross-reacted antibodies, the highly conserved sequence from 17 to 36 amino acid residues was found. The minimal linear epitope from 21 to 30 amino acid residues recognized by broadly neutralized antibody specific to 11 types of HPV, containing Cys22, Lys23, Thr27, Cys28, and Pro29, was inserted into capsid-forming carriers of HPV16 L1, core antigen HPV149,

or nontoxic mutant CRM197 of the diphtheria toxin. The genetic constructs obtained expressed the pan-vaccine in *E. coli* with very high productivity against types of HPV 11, 16, 18, 45, 52, 58, and 59. Thus, the authors announced the broadly neutralized pan-vaccine against HPV with 100% defense and safety (Wang et al., 2015).

3.3 Therapeutic Vaccines

In vaccinology, in more recent times, particular attention has to be paid to the creation of therapeutic types of vaccines that can be applied after preventive vaccines against different diseases, as well as against cancer, if the prevention is weak. Different approaches are employed in these investigations: the irradiation of autologous cancer cells, the use of fragments of pathogen DNA, the introduction of synthetic virus DNA, the inclusion of synthetic peptides that are specific to cancer.

We call therapeutic vaccines those means of medical destination (antigenic proteins or antibodies at the passive immunization) that act on the existing disease or infection, but in very low amounts, because they have the high extent of specificity. Even so, in spite of very intensively conducted investigations, stable results in the use of therapeutic vaccines, perhaps, can be expected in the near future, or farther on. This might be explained by the fact that work on therapeutic vaccines began comparably recently (around 2006–08), as well because of insufficient knowledge about the mechanism of the action of therapeutic vaccines on different diseases.

Increasing the amount of activated T lymphocytes (cell-killers) selectively obliterated tumor cells might be the most expected mechanism of the impact of therapeutic vaccines on tumor cells, upon peroral administration.

As it can be seen in Fig. 10.9, in the beginning, the presentation of antigens on the surface of DC to T cell-killers and T cell-helpers descended by MHC class I and II. Primed CD8+ T lymphocytes (killers) and primed CD4+ T lymphocytes (helpers) destroy tumor cells.

Currently, early genes E2, E6, and E7 are the focus of numerous investigations, and, based on this fact, many attempts are made to create therapeutic vaccines against cervical cancer. It was established that the early protein E2 regulated the transcription on the site of early polyadenylation sign (A_E), meaning that antitermination occurred (Fig. 10.6).

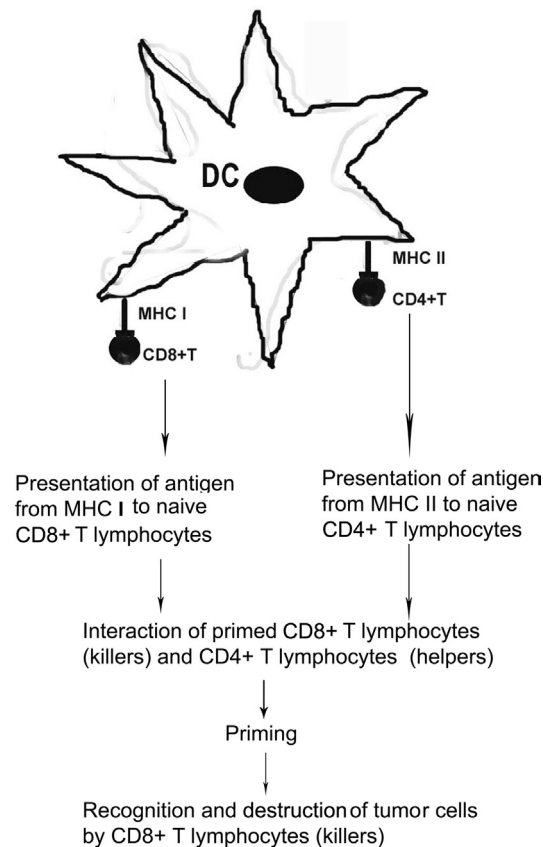


FIGURE 10.9 Scheme of destruction of tumor cells by CD8+ T lymphocytes (killers). *MHC I*, Main complex of histocompatibility class I; *MHC II*, main complex of histocompatibility class II.

This antitermination allowed the polymerase to pass through the site pA_E and increase the transcription of late genes, encoding the synthesis of the capsid proteins L1 and L2 of HPV. The accumulation of large amounts of full-size capsid proteins admitted to process the successful assembly, as well as packaging the DNA genome into capsid, must be specially organized spatially.

The functional role of early protein HPV16 E2 was established in 1985, yet it was only later shown that HPV E2 was the transcriptional regulator of sequences encoded transforming oncogenic proteins E6 and E7. Three sites of binding of E2 were presented in the space region between the site for binding the enhancer and TATA box; and before the site of binding the enhancer there is the fourth site of binding of the protein E2. As a whole, this zone before TATA box was destined as the region of origin and replication and, perhaps, it participated in this process as an essential transcriptional operator, blocking the initiation of replication. Besides this role, protein E2 was known for the role of direct binding of viral genome via transactivation domain of E2 to the chromatin of host cells by means of appropriate kinases. As such, the dual role of E2 protein is proposed in the regulation of cancerogenesis: the blocker of the initiation of replication in *ori* site, and the mediator in holding the ring genome of papillomavirus close to the chromatin (McBride and Jang, 2013).

Proteins of early expression E6 and E7 were named as transforming proteins that directed mammalian cells to the increase of proliferative activity and to neoplasia. Both proteins E6 and E7 have multifunctional activities. However, most characteristic features for the initiation of cancerogenesis are supposed to be the interaction of E6 with p53 protein, called a tumor suppressor, and for the protein E7 the interaction with the protein pRB (retinoblastoma). That is considered a key step in the initiation of cell proliferation because pRB, after joining with E7, lost the connection with eukaryotic factor of initiation of replication e2F and liberates it. The activation of cyclin-dependent kinase (cdk) occurs; and the cell cycle shifts to the stage of DNA synthesis. Protein E7 is expressed in the culture of prime cells of CIN, keratinocytes that acquired the behavior of immortality, and proliferated as immortal culture of keratinocytes.

The E7 protein is small, 98 amino acids in size, 11 kDa, and it has the structure of zinc-finger that is the evidence of its function as a factor of transcription. From the other site, in spite of its small size, there are 46 epitopes in the structure of E7 protein that are evidence for its capability to evoke the immune response with formation of neutralizing antibodies. Due to the manifestation of the presence of epitopes in E7, there was proposed the role for E7 protein as a therapeutic vaccine, as early as the 1990s because the protein E7 induced the synthesis of specific antibodies after the immunization of mice. As E7 did not reveal special selectivity to HPV types, it was considered that E7 was a possible candidate for the creation of vaccines against many types of papillomaviruses on its base.

In the early 1990s–2000s, E7 was obtained mainly in *E. coli* cells or in baculoviral expression system. The synthesis of E7 protein was abundant, therefore molecules of E7 assembled in dimers, trimers, and oligomers of more complicated order associated in particles. The experiments in the study of the immune activity of E7 were mostly negative and, in order to increase the immune response, adjuvants were added. Thus it was proposed that E7 was inactive in immune response; that was in contradiction with the presence of many epitopes, both linear and conformational in E7 structure.

After the establishment of the fact of binding E7 to pRB, the whole series of investigations was followed with the confirmation of cancerogenesis after injection of E7 solution into mice. Therefore, it was believed that E7 was responsible moreover for tumorigenesis, followed by infection with high oncogenic types of papillomaviruses.

During 2007–09, in work by Italian scientists together with V. Yusibov (USA), it was shown that the application of E7 received in plant virus expression system developed in tobacco plants resulted in the regression of lymphomas, as visible as decreasing in size and disappearance (Venuti et al., 2009). Authors announced the usage of E7 from tobacco leaves as candidate therapeutic PMV against tumorigenesis.

In 2011, the dual role of E7 HPV was proposed: (1) the induction of tumorigenesis, (2) the induction of synthesis of specific neutralizing antibodies, and the rejection of tumors. Mice were given three times, in an interval of 2 weeks, 10 mkg of E7 protein; 2 weeks later after the last administration, mice were injected with tumor cells TC-1. However, the mice did not form tumors, although the controls formed tumors after injection of TC-1. If mice were vaccinated only with one dose and TC-1 were introduced afterward, there were small tumors appearing that were indicated only by careful palpation (Petrone et al., 2011). Authors naturally concluded that, due to dual role of E7 protein in tumorigenesis, E7 might be a successful model for the creation of therapeutic vaccine against cancer.

At the present time, there a large amount of the clinical trials of E7 are conducted in different combinations with E6 and E2, and with use of many variants of adjuvants, DNA vaccines, and E7 peptides from several sources, because a small size of it also allows to obtain E7 by synthesis in different expression systems, and by alive viral vector.

As far as we are aware, even healthy mice infected with high oncogenic types of papillomaviruses were able to clear the papillomaviruses during 1 year, so that the issue of regulation of events leading up to cancerogenesis after infection and

otherwise to therapy of the existing cancerogenesis was concluded mostly in the state of immunoreactivity and the function of immune system as a whole. Therefore, this issue is still very complicated so far and needs further investigations.

The production of E7 protein, as shown in the work of Rybicki and coauthors (Whitehead et al., 2014), was low for the reason of instability in tobacco plants. If the E7 gene encoded E7 protein (without binding pRB site, 7GGGG) was introduced into the genetic construct pUC18ZERA with self-assembled domain of γ -zein protein from corn, and introduced by agrobacterial transient transformation, so then the production of target protein E7 was composed of as much as 0.6–1 g/kg tobacco leaves. The presence of the antisilencer sequence in the construct of plasmid pUC18 ZERA facilitated to the sharp increase in the amount of target protein. If only the component of ZERA revealed the immunogenic activity, otherwise, as a whole, the complex body ZERA-16E7 synthesized in tobacco leaves led to the regression of tumors in mice, therefore the candidate therapeutic vaccines against cervical cancer can be created based on this complex (Whitehead et al., 2014).

Successful clinical trials of PMV on volunteers were conducted during the pandemic of influenza A H1N1 in 2009, in the United States. The recombinant antigenic protein hemagglutinin HAC-1 of influenza virus A H1N1 was produced in plant virus expression systems on tobacco plants with the use of TMV replicase (Jul-Larsen et al., 2012). A number of 28 volunteers were injected in the deltoid muscle with 0.5 mL of preparation of HAC-1 solution purified from extracts of tobacco leaves. The analysis of blood serum and lymphocytes indicated the presence of specific neutralizing antibodies, and the increase in the level of interleukin IL-2 and interferon.

In 2014, we began our work with oncogenic HPV16 E7 and developed the construct for the creation candidate vaccine for expression in tomato fruits transformed with combined agrobacterial plant viral system. The first experiments showed that the created genetic construct provided the expression of target gene *hpv16* E7 and the synthesis of antigenic protein HPV16 E7 in transgenic plants upon agrobacterial transformation.

Summing up all statements, it might be concluded that innovative biotechnologies employing methods of molecular biology and genetic engineering opened new possibilities of creation of new types of preventive and therapeutic vaccines, for whose elaboration researchers worked intensively in laboratories in many countries.

3.4 Plantibodies as Therapeutic Vaccines

The syntheses of full-sized antibodies and antibodies of reduced size (miniantibodies, single chain fragments of antibodies) are very expensive events that require, besides financial support, the bulk of serious knowledge in molecular biology, genetics, biochemistry of proteins, and immunology. Therefore, the most suitable methodology of recombinant biotechnologies employing expression vectors placed in one or other expressive systems has appeared. It was found that, in bacterial or yeast expression systems, only the simple and mostly diagnostic minibodies or Fab fragments carrying immunodeterminants could be obtained. But it was not possible to synthesize functionally active full-sized antibodies that reacted with complement proteins in bacterial or yeast expression systems because there was no appropriate folding, as well as due to the insufficient isomerization and the absence of the glycosylation proper to mammals.

Only plant expression systems turned out to overcome these disadvantages, and were fortunately able to synthesize most exact antibodies as the cells of mammals culture did, in order to provide the correct folding, isomerization, and glycosylation (Hiat and Pauly, 2006).

Monoclonal antibodies are used in two directions: (1) as a means for passive immunization with high specificity and (2) as microbicide remedy applied to the supposed place of infections.

Monoclonal antibodies began to be produced in corn seeds, in rice seeds, and in the seeds and leaves of Arabidopsis. The produced antibodies were as effective as monoclonal antibodies formed in mammalian cell culture or in CHO (cells of hamster ovaries). Taking into account their easier scalability, the cost of monoclonal antibodies produced in plants (plantibodies) is suggested to be 10 times lower (Ma et al., 2015).

By means of recombinant technologies, highly effective broad neutralizing antibodies against HIV (2G12) were created in CHO. It was considered that necessity for this antibody will be 1000 kg/year, for both therapeutic and preventive means, in order to control the expansion of HIV in the world. Mammalian cell cultures or CHO are not able to provide the production of such large amount of these antibodies. Ma et al. (2015) suggested to employ the plant virus expression system for the production of this microbicide on the base of tobacco plants that produced 10 mkg/g of fresh weight in the first generation of plants and 25 mkg/g of fresh weight in the second generation of plants. Clinic trials revealed that microbicide on the base of plant-derived antibody was not toxic for women even if it was applied in amounts of 28 mkg intravaginally.

Using the replicase of bean yellow mosaic virus (BeMV) with DNA genome, the expression system for the synthesis of the EIC against Ebola virus was created in tobacco leaves (Bhoo et al., 2011). According to the design of the genetic

construct, the glycoprotein of Ebola virus was connected to the C terminal of heavy chain of humanized monoclonal antibody 6D8 IgG that specifically joined to linear GPI. The construct was placed into agrobacterial vectors for transformation. The rate of the synthesis of antibody was high and, after agroinfection in 4–6 days, it accumulated up to 40 mkg/g of fresh weight of tobacco leaves. The dose of 10 mkg EIC with Ebola glycoprotein was injected into linear mice or in guinea pigs that gave 100% defense against Ebola virus. However, there were no trials on humans or primates. Charles Arntzen published in 2015 a study in which it was shown that EIC from tobacco leaves was successfully applied on volunteers at the time of the Ebola epidemic in Africa in 2014, when more than 10,000 people died from this disease. These scientists have also shown successfully that only antibodies with glycosylation were active in joining with the Ebola virus (Arntzen, 2015).

At the present time, a large number of investigations were carried out with glycoengineering of plantibodies in order to elicit the posttranslational modification of mucosal antibodies with biosimilars to human glycosylation, with the final purpose to succeed in mucosal passive immunization with IgA and IgM. These works are mostly directed to commercial exploitation of plant expression systems for the creation of biopharmaceuticals for passive immunization against many infectious diseases (Vasilev et al., 2015). But, as far as the final critical milestone must be trials on humans, these investigations are still at the stage of the research of attempts to decide on the physical-chemical and pharmacological peculiarities of glycosylation of mucosal antibodies.

Passive immunization by antibodies against plant pathogens is considered for exploitation in order to overcome the bare patch of *Rhizoctonia solani* and other pathogenic excitors of plant diseases.

4 FUTURE PERSPECTIVES

Plant transient expression system is currently declared to be the most mobile and reliable system for the development of medicaments that might be turned to scalability very successfully, compared to any of the existing recombinant expression systems. By employing this system, as much as 1000 kg of vaccine biomass/year with the content of target antigenic protein of 5–10 mkg per 1 g of fresh weight can be produced. The necessity of large production of antigenic proteins or antibodies appears during outbreaks of diseases or epidemics (e.g., the Ebola virus, cholera, leishmaniosis), and with the threat of bioterrorism.

In spite of highly evidenced advantages of PMV, only one vaccine against cholera received FDA approval and, perhaps, will be applied to people (e.g., in Iraq). At the first stages of clinical trial are PMV against influenza (both pandemic and seasonal), rabies, Norwalk virus, hepatitis B (Table 10.2).

The conflicts of interests from the authorities on classical injected and nasal-pharyngeal vaccines make a reason for the slow introduction of PMV to the population, with the aim of prophylaxis. Therefore, the demands ascertained for PMV were mechanically transferred from those belonging to classical injected vaccines. There are: (1) dosage, (2) preparation of vaccine and its formulation, (3) administration, (4) transportability, (5) storage, (6) stability to temperature impact, (7) schedule of vaccination, (8) usage of adjuvants. Besides this, it is necessary to continue the study of optimization of glycosylation of vaccines by using UDP-transferase with broad specificity.

Perhaps in the future it would be advisable to apply consequent preventive followed by therapeutic vaccinations, if the preventive vaccination would not be satisfied. The investigations in this direction seem to be of great importance.

TABLE 10.2 Vaccines on Plant Base With Ongoing Clinical Trials in Humans

Product	Disease	Plant	Stage of Trial
H1N1 HA VLP	Pandemic influenza A	<i>Nicotiana benthamiana</i>	Stage I/II
H1N1 HA VLP	Seasonal influenza A	<i>Nicotiana benthamiana</i>	Stage I
NVCP	Norwalk virus	Tobacco, potato	Stage I
HBsAg (HBV) VLP	Hepatitis B	Tobacco	Stage I
HBsAg (HBV) VLP	Hepatitis B	Tobacco	Stage I
Alfalfa mosaic virus with glycoprotein of rabies virus	Rabies virus	Spinach	Stage I
Glucocerebrosidase	Gausher disease	Carrot tissue culture	FDA approved
MucoRice B-subunit CTB	Cholera	Rice seeds	FDA approved

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