REVIEWS

State of Research in the Field of the Creation of Plant Vaccines for Veterinary Use

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Abstract—Transgenic plants as an alternative of costly systems of recombinant immunogenic protein expression are the source for the production of cheap and highly efficient biotherapeutics of new generation, including plant vaccines. In the present review, possibilities of plant system application for the production of recombinant proteins for veterinary use are considered, the history of the “edible vaccine” concept is briefly summarized, advantages and disadvantages of various plant systems for the expression of recombinant immunogenic proteins are discussed. The list of recombinant plant vaccines for veterinary use, which are at different stages of clinical trials, is presented.

Keywords: transgenic plants, plant vaccines, biosafety, expression systems, recombinant proteins, veterinary

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INTRODUCTION

For many thousands of years plants have served humanity as a source of medicinal substances. However, only at the turn of the century XXI with the appearance of DNA technologies, it became possible to modify plant genomes and to create new types of plants (transgenic plants), which are capable of synthesizing and accumulating in their tissues recombinant proteins from various heterologous systems. To date transgenic plants in which nuclear and chloroplast genomes have been transformed with genes encoding heterologous proteins that are important in the treatment of various diseases — antigens of infectious agents, antibodies, immunomodulators, etc. have been created [1, 2]. Of principal importance of this work development was the creation of the “edible vaccine” concept, the essence of which is the use of genetically modified plants containing protein antigens of infectious agents for oral delivery of relevant antigens to the mucosa of the gastrointestinal tract of warm-blooded animals.

Vaccination based on the programming of the specific mechanisms of warm-blooded animal protection against pathogens is the most efficient method for the struggle against infectious diseases, which often result in a mass mortality. In agriculture, there is no alternative to livestock vaccination, because there are no antiviral drugs that are suitable for a wide use in animal husbandry. The importance of animal vaccination

indirectly affects human health, because the use of vaccines significantly reduces the amount of pharmaceuticals in the food chain.

As a rule, animal immune mechanisms are activated by the direct introduction of infectious agents or their components. At present, most of used vaccines are preparations on the basis of inactivated agents. Although these vaccines manifest the high immunogenicity, they are not without serious shortcomings. Among such disadvantages are the increased sensitivity of the organism to them, the large load on the immune system, the reactogenicity of vaccines (side effects), their toxicity, etc.

The application of molecular biology and genetic engineering methods opened wide prospects for the manufacturing of vaccines of new generation, which immunogenic components can be biological molecules or their fragments. DNA fragments or proteins of the infectious agent cell envelopes can serve as immunogenic components. When the gene encoding the envelope protein of the infectious agent is transferred into the genome of another organism, for example, plant, then the cells of such plant will synthesize the protein antigen capable of formation of resistance to this agent. Thus, the introduction into the organism not the whole pathogen but only its part, which is not capable of inducing infection development, will provide for the effect of vaccination.

Manufacturing of medicinal preparations for veterinary use is a very important and dynamically developing field of world industry. Vaccine production is about

Abbreviations: TMV—tobacco mosaic virus; TSP—total soluble protein.

one fourth of the total pharmaceutical market of drugs for veterinary use, and it is constantly expanding. [3].

Preparation of medicinal substances for the production of veterinary products is based on various approaches, including biotechnology using genetically modified (transgenic) organisms for these purposes; such expression systems as bacteria, yeast, cells of insects and mammals are used. The application of genetically modified plants with genes encoding pharmaceutical proteins inserted in the genome opens new prospects for obtaining recombinant proteins, including plant vaccines [4].

This review is devoted to the analysis of possibilities of producing recombinant immunogenic proteins for veterinary use on the basis of plant expression systems, and the history of the concept of “edible vaccines” for animal immunization.

BRIEF HISTORY OF RESEARCH DEVELOPMENT ON CREATION OF PLANT VACCINES

The idea of the usage of plant cells for the synthesis and accumulation of recombinant protein antigens was for the first time successfully realized in 1992 by C. Arntzen and his colleagues [5]. Just this team of researchers not only demonstrated a possibility of the accumulation of the surface HBsAg antigen of hepatitis B virus but also its capability for self-assembling in the virus-like particles in transgenic tobacco plants. The virus-like particles isolated from plant tissues were identical to the particles of HBsAg antigen of industrial recombinant vaccine obtained in the yeast expression system and also to virus-like particles from the blood plasma of patients infected with hepatitis B virus. Thus, it became obvious that genetically modified plants producing and accumulating protein antigens of various infectious agents can be used for oral delivery of corresponding agents to the mucosa of the gastrointestinal tract of warm-blooded animals, i.e., as “edible vaccines.”

The next important step in the development of the “edible vaccine” concept on the basis of genetically modified plants was the creation of transgenic plants producing the heat-labile enterotoxin of *Escherichia coli* [6, 7] and B-subunit of the cholera toxin [8]. Heat-labile toxin of *E. coli* consists of two parts: LT-A (enzyme) and LT-B (pentamer of receptor-binding polypeptides). LT-B binds to the receptors on the surface of membranes of epithelial cells of the mammal small intestine and transports LT-A in the intestinal cells, where it induces changes in the cell metabolism and cell dehydration. When the two parts of the heat-labile enterotoxin are separated, the appearance of the LT-B protein complex on the surface of epitheliocytes will stimulate a strong immune response of intestine mucosa without the appearance of any disease signs. Just this feature was the basis for the research of C. Arntzen [6] team on the creation of plant vaccine

providing for the resistance to enterotoxigenic *E. coli* toxins. The authors established that LT-B synthesized in transgenic tobacco and potato plants and also LT-B isolated from *E. coli* delivered orally to mice induced similar immune responses.

Later, LT-B sequence was optimized for expression in plant cells and transferred into the potato genome [7]. In potato tubers, the protein assembled correctly in oligomers and was accumulated in amounts sufficient for the induction of the immune response at oral delivery to the organism. On the basis of clinical trials of the “candidate” plant LT-B vaccine, it was established that the consumption of raw potato tubers containing 0.3–10 mg LT-B by volunteers resulted in the formation of serum and mucosal immune responses with high titers of antibodies [9].

The initial concept of “edible vaccine” was heavily criticized by researchers who believed that in the aggressive medium of the gastrointestinal tract the recombinant protein should be destroyed. However, later it was experimentally established that the recombinant B-subunit of the cholera toxin fused with green fluorescent protein (GFP) protected by the plant cellulose cell wall at oral delivery is capable of passing through the gastrointestinal tract and reaching the antigen-containing cells of the mouse intestine [10]. The results obtained confirmed the possibility of using plants synthesizing protein antigens of various infectious agents for oral delivery of antigens to the mucosa of the gastrointestinal tract and experimentally confirmed a consistency of the “edible vaccine” concept. It became evident that genetically modified plants can be used for the creation of plant vaccines as a raw material, and separate plant parts (fruits, roots, berries, leaves, etc.) can be used directly in food without preliminary heat treatment.

MECHANISM OF MUCOSAL IMMUNE RESPONSE FORMATION

In the process of evolution, mammals developed secondary lymphoid mucosal tissue capable of antigen absorption, processing them, and using for the induction of the mucosal response. It was established that in this case both cellular and humoral immunity were formed. It is of importance that adaptive mucosal immunity can distinguish between usual food and symbiotic antigens and infectious agents [11]. The scheme of the mechanism of mucosal immune response formation is presented in Fig. 1.

In the digestive tract, which is one of the pathways for the penetration of a variety of pathogens into organism, the main associated lymphoid tissue is the Peyer's patches. Peyer's patches, inductive sites of the intestine, which contain the dome, underlying the follicle (B-zones with germinal center) and interfollicular region containing T-cells. The surface of the dome is covered by a specialized follicle-associated epithelium containing the folded cells (M-cells), which are

Fig. 1. Scheme of mucosal immune response formation (cyt. after [12] with modifications).

Permanent immune surveillance is carried out by specialized antigen-transporting cells (M-cells) and antigen-receiving dendritic cells (1). Dendritic cells are the mediators in development of various subpopulations of T-helper cells from naive T cells (2) and activate transcription factors and cytokines. Mature T-helper cells then return to the mucosal surface to function as effectors (3). T-helpers 17 expressing IL-17 activate expression of the receptors of polymeric immunoglobulins (pIg) (4). In their turn, soluble cytokines secreted by dendritic cells can initiate T-independent switching over the synthesis of immunoglobulins (5).

able to absorb and transport antigens from the intestinal lumen. After successful capture, the antigen is partially cleaved and enters into dendritic cells (see Fig. 1, step 1). Dendritic cells are especially important in the initiation of adaptive immune responses, since they migrate to the lymph nodes (see Fig. 1, step 2), and the

mediators act in the development of various subpopulations of T-helper cells from naive T-lymphocytes and also can interact with B-lymphocytes (see Fig. 1, step 5). The activated B- and T-cells leave the Peyer's patches and penetrate into the circulatory system. Mature T-helper cells then return to the mucosa sur-

face to function as effectors (see Fig. 1, step 3). T-helper 17 expressing interleukin-17 (IL-17) increases the expression of the receptors of polymeric immunoglobulin (pIg) and secretion of antigen-specific IgA (Fig. 1, step 4). Subsequent generation of mature plasma cells producing IgA leads to the induction of antigen-specific protection of local and distal mucosal surfaces.

Since the mucosal immune response has a generalized nature, oral (i.e., mucosal) vaccination is not only the immune response of the mucous membranes, but also the overall immune response of the organism [11–14]. It is proved that vaccination via the surface of mucosa membrane can specifically activate the immune response to infection without the development of such processes related to the disease as inflammation or toxicity [14, 15].

ADVANTAGES AND DISADVANTAGES OF PLANT VACCINES

In comparison with traditional expression systems, plant systems are attractive to researchers in many ways, primarily due to the absence of the risk of plant cell infection with animal pathogens, viruses, prions, etc. Plants are capable of the synthesis of most recombinant antigens with the same posttranslational modifications as in animal cells [16]. Plant vaccines can play especially important role in the protection of animals against diarrheal diseases and diseases, which infectious agents penetrate into the organism through the mucosal tissues.

Modern techniques of genetic engineering allow to selectively direct the recombinant proteins expressed in plant cells to various plant organs (seeds, tubers, fruits, etc.) [17]. This possibility greatly simplifies the large-scale production of plant vaccines and reduces their cost. According to the experts, the final price of the product (recombinant protein) produced in a plant expression system will be much less than the price of a similar protein produced, for example, in mammalian cell culture [18].

Since recombinant proteins can be accumulated in the storage organs or seeds, they are able to be maintained without any changes and the loss of biological activity for a long time (months and years). It was established that the recombinant protein of cholera toxin B subunit remained stable in transgenic rice grains for at least 18 months, when grains were stored under room temperature conditions [19]. Recombinant protein antigens remained stable in rice grains during three years and provided for the formation of the protective immunity in mice against cholera agent or against enterotoxigenic *E. coli* [20]; in soybean seeds and soy milk, the preservation of antigen stability was observed for four years [21]. Thus, grains of transgenic plants can be transported to the site of final destination without additional freezing and treatment, and this ensures the retention of activity of the recom-

binant protein activity, its stability, and the constancy of dosage.

A somewhat different picture is observed when lyophilization is used as a method of the conservation of protein antigens synthesized by plant cells. It was established that the recombinant protein of the norovirus envelope retained its immunogenicity in tissues of both lyophilized and air-dried tomato fruits [22], but the tested samples differed in immunogenicity. The immunogenicity of this recombinant protein in air-dried tomato fruits was somewhat higher in comparison with lyophilized fruits. Similar results were obtained in experiments on the lyophilization of potato tubers synthesizing the protein of norovirus envelope, e.g., the immunogenicity of lyophilized tubers was lower than that of fresh tubers [22]. However, additional studies are required for the final solution of this question.

Despite these advantages, plant vaccines are not without disadvantages. One of them is differences in protein posttranslational modifications in plants and animals, e.g., in glycosylation of the recombinant protein [23]. It is known that more than a half of proteins synthesized by eukaryotic cells are glycosylated and more than a third of currently applied biopharmaceuticals are glycoproteins [24]. Although the activity of most proteins does not depend on glycosylation, in some cases it may be critical. Specific features of protein glycoforms can affect their folding, stability, transportation, and changes in their functional activity and immunogenicity. Examples of biopharmaceuticals, which functional activity depends on the specific glycoform, are erythropoietin, antibodies, blood antigens, some interferons and hormones [24].

Scheme of the N-glycan complex formation in plants and animals (humans, for example) is shown in Fig. 2. The most significant difference in glycosylation is that the plant β -1,2-xylose is attached to the core mannose residue and α -1,3-fucose — to N-acetylglucosamine residue of the core glycan. In human cells xylose is not used at all in glycosylation and a proximal fucose residue is attached to glycans through the α -1,6-bond. It was established that sugar residues attached at posttranslational modifications of recombinant proteins in plant cells themselves were capable of exhibiting the immunogenicity. Approximately in one quarter of patients with allergy symptoms, IgE-antibodies specific for complex glycans, which include xylose or fucose, were revealed [25]. Differences in glycosylation during posttranslational modifications of recombinant proteins in plant and mammal cells can be eliminated by genetic engineering techniques, which was successfully demonstrated on the moss *Physcomitrella patens*, in which the genes encoding the enzymes β -1,2-xylosyltransferase and α -1,3-fucosyltransferase responsible, respectively, for xylosylation and fucosylation of proteins were switched off by the “knock-down” method [25].

Thus, performed studies allow a suggestion that the plant vaccine based on the genetically modified plants is capable of inducing protective immunity and open new opportunities for the creation of low-cost and easy handling vaccines against infectious diseases of animals. It is of importance that developed to date, highly effective methods of cultivation of agro-economically important plant species, as well as the seed production system for a particular culture make the plants attractive to be used as “biofactories” for manufacturing cheap recombinant proteins for medical purposes.

Due to the rapid development of genetic engineering, to date researchers have developed several systems of expression of target genes in plant cells. The plants stably expressing a heterologous gene and passing it from generation to generation are used most often. Such systems include transgenic plants (target gene is inserted into the nuclear genome) and transplastome plants (target gene is inserted into the genome of chlo-

roplasts). Among plant expression systems with stable integration of the transgene in the nuclear genome, a separate group includes the cultivated duckweed, microalgae, and cell culture systems in vitro: cell suspensions, cultures of “hairy roots”, moss protonemas, which cultivation conditions are a completely closed environment (bioreactors). Promising is the transient expression system, in which the target gene is introduced into plant cells and is expressed for a short period of time (several days), but is not integrated into the genome. Each of these expression systems has its advantages and disadvantages; the main details of these systems are considered below.

Transgenic Plants

The most widely used system of heterologous gene expression, in particular for plant vaccine production, are transgenic plants with the stable integration of the transgene into the nuclear genome. The creation of these plants involves the transfer of foreign genes into the genome of plant cells using *Agrobacterium tumefaciens* or bioballistics and subsequent regeneration of transformed plants from these cells. Being inserted into the nuclear genome, transgene becomes its resident part, is stably expressed, and is maintained in subsequent generations.

Using this expression system for producing recombinant proteins, including plant vaccines, is still hampered by relatively low levels of transgene expression, which is, as a rule, less than 1% of total soluble protein (TSP) and also by its variability in different plant organs and tissues within a single plant and in different plants. Most often, the variability in expression of the transgene is due to the random nature of its insertion into the nuclear genome (effect of position) and may be associated with partial or complete transgene inactivation [27, 28]. Experts believe that the use of plant expression systems for obtaining plant vaccines is economically beneficial at the level of expression of a target gene, which allows the accumulation of recombinant protein in an amount not less than 1% of TSP [29].

A lot of ways to increase the level of foreign gene expression in transgenic plants is developed; they are very fully discussed in the reviews [17, 30, 31]. Among them are the optimization of the codon composition of the target sequence, the usage of strong promoters, the addition of introns or regions of binding with the nuclear matrix (SAR), and others. The search for tissue- and organ-specific promoters, i.e., promoters providing for target gene expression in definite plant tissues or organs, is of a special interest. For example, the usage of promoters directing transcription of the target gene predominantly in the seeds can increase the yield of the target protein by an order or several-fold: up to 13–14% of TSP in rice grains and up to 25% of TSP in tobacco seeds [17]. It is of importance that as compared with leaves, seeds contain less pro-

teases and much less water; therefore, recombinant protein is saved better.

Despite the fact that transgenic plants with stable transgene expression in the nuclear genome are used most widely, it is just this expression system that induces a cautious attitude of human society. One of the fears is associated with the possibility of transgene transfer from the cross-pollinated plants into the genomes of wild relatives at growing biotechnological crops in open field. To solve this problem, researchers developed various agricultural technologies as well as fixing male sterility in transgenic plants to prevent unwanted cross-pollination of cultivated and wild species. Examples of production of various immunogenic proteins using for this purpose transgenic plants with stable transgene integration into the nuclear genome are presented in Table 1.

Transplastome Plants

Chloroplasts are most attractive among plant expression systems. Genetically modified plants with the stable transgene integration into the chloroplast genome were called transplastome plants. The specific organization of chloroplasts allows achieving a high dose of foreign gene in transplastome plants, which provides for the efficient production of the target protein. The record of the recombinant protein yield was achieved by transplastomic tobacco plants with the bacteriophage lysin gene *PlyGBS*, encoding a hydrolase of the bacterial cell wall, the level of which accumulation in the leaves amounted to 70% of TSP [32]. Although in some cases, negative physiological changes were observed in plants with such high level of foreign gene expression, usually there were no deviations in the development of such plants. Problems of transplastome plant adaptation to the high level of foreign gene expression are discussed in the review of Bally et al. [33].

Transgene delivery to chloroplasts is performed using bioballistics (gene gun); its integration into the chloroplast genome occurs via homological recombination [34]. The advantage of chloroplast expression system is in the absence of the effect of position observed in the case of random pattern of transgene distribution in the nuclear genome. In plastids, there is no transgene splicing (inactivation); therefore, its expression is stably preserved in subsequent generations. Due to the prokaryotic organization of chloroplast genome, there is a possibility of co-expression of several genes within a single operon [35]. An important specificity of plastids is that they are inherited through the maternal line and usually are absent from pollen. Therefore, as distinct from usual transgenic plants, transplastome plants are safe for environment because the uncontrolled spread of the transgene into other plants is prevented [36, 37].

It should be noted that protein posttranslational modifications, e.g., assembling multimer proteins, the

Table 1. Plant vaccines for animals

Pathogen	Antigen	Method of introduction	Plant species	Expression level	Immunogenicity of recombinant protein in vivo	Source
Group A rotavirus of cattle	Protein VP6	<i>Agrobacterium tumefaciens</i>	<i>Solanum tuberosum</i> cv. Mayqueen	0.01% TSP	Not examined	[68]
		Potato X virus	<i>Nicotiana benthamiana</i>	5% TSP	Not examined	[69]
		<i>A. tumefaciens</i>	<i>S. tuberosum</i> cv. Bintje	0.01% TSP	(+) mice	[70]
	Protein VP4	<i>A. tumefaciens</i>	<i>Medicago sativa</i>	0.01% TSP	(+) mice, orally and intraperitoneally	[71]
Bovine herpesvirus BHV-1	Glycoprotein D	TMV	<i>N. benthamiana</i>	15–20 µg/g fresh leaves	(+) mice and cattle, intramuscularly and intranasally	[72]
Foot and mouth disease	Protein VP1	<i>A. tumefaciens</i>	<i>M. sativa</i>	—	(+) mice, parenteral	[73]
		Bamboo mosaic virus	<i>Chenopodium quinoa</i> , <i>N. benthamiana</i>	200–500 mg/g fresh leaves	(++) swines	[59]
	Proteins VP1 and VP7	<i>A. tumefaciens</i>	<i>Stylosanthes guianensis</i> cv. Reyan II	0.1–0.5% TSP	(+) mice	[74]
Rinderpest virus of cattle	Hemagglutinin H	<i>A. tumefaciens</i> , bioballistics	<i>N. tabacum</i>	up to 0.75% TSP	(+) mice	[75]
			<i>Arachis hypogaea</i>	up to 0.50% TSP	(+) mice	[76]
		<i>A. tumefaciens</i>	<i>Cajanus cajan</i> sp.	0.49% TSP	Not examined	[77]
Canine parvovirus (CPV)	Peptide from protein VP2	<i>A. tumefaciens</i>	<i>Arabidopsis thaliana</i>	1.2% TSP	(+) mice	[57]
Transmissible gastroenteritis virus of swines (TGS)	Protein S	<i>A. tumefaciens</i>	<i>Zea mays</i>	2% TSP	(++) swines	[55, 62, 63]
		<i>A. tumefaciens</i>	<i>S. tuberosum</i> cv. Desirée	0.02–0.07% TSP	(+) mice	[78]
		<i>A. tumefaciens</i>	<i>N. tabacum</i>	0.1–0.2% TSP	(+) swines	[64]
		<i>A. tumefaciens</i>	<i>N. tabacum</i>	more than 0.1% TSP	not examined	[79]
Hemorrhagic fever virus of rabbits	Protein VP60	<i>A. tumefaciens</i>	<i>S. tuberosum</i> cv. Desirée	6.5–18 µg/g fresh leaves	(+) rabbits	[65]
	Protein VP60	<i>A. tumefaciens</i>	<i>A. thaliana</i> , ecotype Columbia	0.1–0.8% TSP	(+) mice	[80]
	Protein VP60	<i>A. tumefaciens</i>	<i>N. tabacum</i> cv. Petit Havana SRI, <i>S. tuberosum</i> cv. Albatros, <i>Pisum sativum</i> cv. Greenfeast, <i>Brassica napus</i> cv. Westar	Tobacco 0.002% TSP; pea 0.005–0.002% TSP; potato, canola, less than 0.00001% TSP	(++) rabbits	[66]

Table 1. (Contd.)

Pathogen	Antigen	Method of introduction	Plant species	Expression level	Immunogenicity of recombinant protein in vivo	Source
Newcastle disease virus (NDV) of birds	Proteins F and NH	<i>A. tumefaciens</i>	<i>S. tuberosum</i> cv. Ken-nebec	0.03–0.06% TSP	(+) mice	[81]
	Glycoprotein F	<i>A. tumefaciens</i>	<i>Z. mays</i>	0.9–3% TSP	(++) chickens	[56]
		<i>A. tumefaciens</i>	<i>Oryza sativa</i>	0.25–0.55% TSP	(+) mice, intraperitoneally	[82]
Avian infectious bronchitis	Glycoprotein S1	<i>A. tumefaciens</i>	<i>S. tuberosum</i> cv. Dong-nong 303	0.07–0.22% TSP	(++) mice and chicken	[83]
Avian influenza (H5N1)	Hemagglutinin M2	CMV	<i>N. benthamiana</i>	0.1% TSP	Not examined	[84]
	Hemagglutinin rHA0	magnICON, TMV	<i>N. benthamiana</i>	0.3 g/kg fresh leaves	(++) chickens, intramuscularly and subcutaneously	[85]
Japanese encephalitis virus (horses, cattle, pigs)	Glycoprotein E	<i>A. tumefaciens</i>	<i>O. sativa</i> cv. Nipponbare	0.011–0.019% TSP	(+) mice, intraperitoneally	[58]
Porcine reproductive and respiratory syndrome (PRRS)	M-protein	Bioballistics	<i>Z. mays</i>	5.1 µd/g fresh callus	(+) mice	[86]
Swine fever	Glycoprotein E2	Potato X virus	<i>N. benthamiana</i>	120–150 mg/g fresh tissues	(+) rabbits	[60]
Rabies	G-protein	<i>A. tumefaciens</i>	<i>Lycopersicon esculentum</i> var. UC82b	0.01–0.1% TSP	Not examined	[87]
		TMV	<i>N. benthamiana</i> , <i>Spina-cia oleracea</i>	0.3 mg/g fresh tissues	(+) mice, orally and intraperitoneally	[88]
	G- and N-proteins	TMV and alfalfa mosaic virus	<i>N. tabacum</i> cv. Samsun NN, <i>N. benthamiana</i> , <i>S. oleracea</i>	0.3 ± 0.03 mg/g fresh tissues	(+) mice, orally and intraperitoneally	[61]
	G-protein	Bioballistics	<i>Z. mays</i>	1% TSP	(++) mice (++) sheep, 83%	[89, 90]
	N-protein	<i>A. tumefaciens</i>	<i>L. esculentum</i> , <i>N. benthamiana</i>	Tomato 1–5% TSP; Tobacco 45% TSP	(++) mice, some did not die	[91]
	G-protein	Bioballistics	<i>Daucus carota</i> var. Ren-didora	0.4–1.2% TSP	(++) mice, 66%	[92]

TMV—tobacco mosaic virus; CaMV—cauliflower mosaic virus; TSP—total soluble protein; (+) immune response is revealed at oral immunization; (++) immune response is revealed at oral immunization; animals did not die after virus infection. Species of immunized animals are indicated, the way of antigen delivery is indicated in the cases when it was not oral; the amount of survived infected animals is indicated in the cases when the survival was less than 100%.

formation of disulfide bridges, lipid modifications, etc., occur successfully in chloroplasts. It is established that recombinant proteins synthesized in chloroplasts do not differ from native ones in their functional activities [38, 39]. Chloroplast attractiveness as the system of recombinant protein expression is in the fact that they are closed structures and it preserves the metabolites, which when released into the cytosol are toxic to plant cells, such as B-subunit of cholera toxin [38] and trihalose in tobacco cells [40].

On the basis of the above works, it becomes apparent that transplastome plants can be regarded as the most promising system for efficient production of protein antigens. However, the main disadvantage of such expression system at plant vaccine manufacturing is that chloroplasts cannot glycosylate proteins. At present, the creation of transplastome plants is also associated with some technological problems related to the absence of the efficient system of regeneration for most plant species and also with the absence of the efficient system of transgene delivery to the chloroplast genome providing for the high percent of transformed plant yield.

Suspension Cultures of Plant Cells

The suspension cell cultures on the basis of genetically modified plants attract the attention of researchers as promising potential systems for biopharmaceutics production. Such cultures can be obtained from loose callus tissues induced from genetically modified explants or on the basis of co-culturing of the cell suspension and *A. tumefaciens*. After the assessment of growth characteristics and cell line screening to obtain promising lines capable of the accumulation of great amounts of recombinant proteins, such lines can be cultivated in bioreactors for target protein obtaining.

An attractive feature of the cell cultures as expression systems for recombinant protein production, as compared to the use of whole plants for this purpose, is that cell culture can be unified in growth characteristics, cell dimensions and types. Moreover, cells are grown under strictly controlled conditions, when the product accumulation does not depend on the seasonal weather changes and allows the permanent product obtaining in bioreactors. The additional insertion of signal peptide nucleotide sequences into the construct permits a protein secretion into the intercellular space, which allows target protein isolation directly from the culture liquid. The addition of recombinant protein stabilizers to the suspension culture increases the yield of the target protein [41].

By their capabilities, plant cell cultures are comparable in production of therapeutic recombinant proteins with the conventionally used mammalian cell cultures, such as Chinese hamster ovary cells. However, as distinct from the mammal suspension cultures, they are not infected by any animal pathogens. To date, there are many examples of plant cell cultures

with the yield of recombinant protein in the amount more than 10 mg/L, which is a threshold value for starting the commercial manufacture of the product [42].

An example of a commercially successful production of veterinary vaccine products is developed by Dow Agro Sciences company (United States) system Concert™, patented as an effective and safe system for the production of vaccine proteins in cultured plant cells, cultured in a bioreactor. The first plant vaccine against Newcastle disease virus of birds obtained in the tobacco cell culture was approved for use by the Ministry of Agriculture of the United States in 2006.

The disadvantages of this expression system are still insufficiently high yield of the target recombinant protein and the instability of foreign genes in cultured plant cells due to the epigenetic silencing of the transgene transcription [43]. Advantages, disadvantages, and specific features of recombinant protein production in the plant cell cultures are discussed in reviews [42, 44].

Mosses, Duckweed and Unicellular Algae

There are many examples of successful use of aqueous plants, such as duckweed, unicellular algae, and mosses, which are cultivated similarly as plant cell suspensions in bioreactors, for recombinant protein expression.

Duckweed attracts the attention of researchers as a potential highly efficient system for recombinant protein expression due to its capability of a rapid biomass accumulation: it can be doubled for 24–48 h. Genetically modified duckweed as a potential producer of biopharmaceutic proteins can be used by animals as a raw or dried food. Duckweed is a monocotyledonous angiosperm; for foreign gene transfer into its genome, the methods of agrobacterial transformation and bioballistics are used. Examples are known when genetically modified duckweed accumulated recombinant protein in the amounts up to 25% of TSP, as assessed after the accumulation of GFP [45]. Sequencing the duckweed chloroplast genome is close to completing, which opens up some prospects for a significant increase in the yield of recombinant proteins.

The systems of biopharmaceutics production using genetically modified microalgae are actively developed. Algae combine advantages of both bacteria (rapid growth and simplicity of cultivation) and higher plants (a capability of posttranslational modifications and photosynthesis). *Chlamydomonas reinhardtii* is most promising among algae: it has a short time of biomass doubling (about 10 h), it is easily subjected to nuclear and chloroplast transformation, it can be grown under photoautotrophic conditions or with the addition of acetate as the source of carbon. Nuclear transformants usually give rather low yield of protein product; therefore, recombinant protein production by this alga is based on the transformation of chloro-

plast, which occupies about 40% of the cell volume [46]. *C. reinhardtii* nuclear and chloroplast genomes are sequenced, and this simplifies substantially any genetic engineering manipulations. Known examples of protein antigen production in chloroplasts of *C. reinhardtii* are B-subunit of cholera toxin fused with the coat protein of foot and mouth disease virus [47] or with D2-fibronectin-binding domain of *Staphylococcus aureus* [48], as well as protein-28 virus cryptokaryosis (shrimp disease) [49] and E2-protein of swine fever [50].

The green moss *Physcomitrella patens* is the only representative of bryophytes, the genome of which is currently completely sequenced and approaches to its transformation are developed. The peculiarity of this moss is that at the stage of the haploid juvenile gametophyte (protonema) this moss is morphologically similar to filamentous algae and easily enough cultivated in a bioreactor [25, 42]. Under certain culture conditions the moss can be in the stage of protonema indefinitely long. The attractiveness of this moss species as the system for recombinant protein expression is that, as distinct from plants, fragments of foreign DNA can be integrated in its genome through homologous recombination, which reduces substantially a possibility of transferred gene inactivation. The *P. patens* cells are capable of postranslational modification of proteins of eukaryotic origin. Since at the step of gametophyte the moss has the haploid number of chromosomes, it becomes possible to modify the functioning of individual genes, in particular the moss lines conducting glycosylation of recombinant proteins as in mammalian cell type were obtained [25]. The firm Grenovation (Germany) is developing the technology of biopharmaceutical protein production on the basis of *P. patens* in bioreactors. Expression system for producing biopharmaceuticals based on the green moss is not the part of the food chain and is characterized by a high degree of biosafety.

Transient Expression

As distinct from above described systems based on the stable expression of foreign genes integrated into nuclear or chloroplast genomes, during transient expression target proteins are synthesized in the plant cell during relatively short time (several days) without insertion into the plant genome. At present, the following approaches are used for transient gene expression in plants: gene delivery with the help of agrobacterium, the use of plant virus vectors, and magnification [51–53]. Tobacco mosaic virus (TMV), potato X virus, alfalfa mosaic virus, and cowpea mosaic virus are used as virus vectors [52]. The availability of infectious cDNA clones, the small size of the viral genomes, the short time required for the expression of a target gene, and a high level of expression provides for a high attraction of this system.

The rapid development of this expression system led to substantial modifications of the first gene insertion vectors or full virus vectors, which is a recombinant virus that behaves as wild-type virus but is capable of expressing additional genes. The next step was the creation of “disarmed vectors” (deconstructed vectors) lacking a number of original virus genes, and gene replacement vectors, in which a portion of the viral genes is replaced by alien genes [51]. Viral vectors have several substantial disadvantages: a tendency to the loss of foreign insertion in the process of virus spreading over the plant and a potential risk for environment related to the presence of infectious recombinant viral particles.

Launch vectors represent an alternative to recombinant plant viruses; cDNA of these viruses is delivered to plants within T-DNA region of agrobacterial Ti plasmid. Firstly, primary transcription of T-DNA occurs in the nucleus; then viral RNA is released into the cytoplasm, where its further amplification, translation, and protein synthesis occur [52].

By 2005, the system of agroinfiltration based on the use of plant viruses and agrobacterial binary plasmids was upgraded and named as magnification [54]. At magnification, multiple agrobacterial lines carrying different parts of the TMV genome are used simultaneously. After agrobacterial transfer into the plant cell nucleus, separate parts of viral genome are assembled in plants in the completely functional viral replicon [51]. The substantial modification of the viral genome, including numerous point mutations for the removal of potential sites of splicing, intron insertion, and the removal of the gene encoding envelope proteins, provided for the highly efficient system capable of recombinant protein synthesis (up to 5 g/kg of fresh tissue), which is more than 50% of TSP [51].

Among disadvantages of transient system is a necessity for recombinant protein isolation and purification immediately after its accumulation in the plant, because, as distinct from seeds and fruits, plant leaves and stems cannot be stored for a long time.

The systems of transient expression of recombinant proteins are rather promising in the cases when a rapid production of a small amount of proteins is required. Experiments with transient expression in plants are held indoors, which reduces the risks associated with biosafety to almost zero. Examples of manufacturing immunogenic proteins for veterinary using transient expression systems are presented in Table 1. The systems of transient expression for the production of recombinant proteins are described in more details in reviews [1, 41, 51, 52, 54].

“CANDIDATE” PLANT VACCINES FOR VETERINARY USE

Table 1 presents examples of using various expression systems for the production of “candidate” plant vaccines for veterinary use. Main specific immunogenic

Table 2. Veterinary plant vaccines at different stages of clinical trial

Product	Disease	Plant material	Stage of clinical trials	Developer
Fused proteins comprising epitops of rabies virus	Rabies	Spinach plants	Phase 1 is completed	Thomas Jefferson University, USA
HN-protein of Newcastle disease virus	Newcastle disease of birds	Tobacco cell suspension culture	Approved by USDA*	"Dow Agro Sciences", USA
Mixture of antiviral vaccines	Diseases of horses, dogs, and birds	Tobacco cell suspension culture	Phase 1	"Dow Agro Sciences", USA
Vaccine for birds	Coccidiosis	Canola plants (modified rape)	Phase 2	"Guardian Biosciences", Canada

* USDA—Ministry of Agriculture of the USA.

proteins synthesized at respective diseases (structural proteins, hemagglutinins, glycoproteins) are usually used as antigens. The most commonly used method of transgene construct delivering into plant cells is still the agrobacterial transformation. In some cases, the level of target protein expression was rather high [55–58], especially in the systems of transient expression [59–61], and suitable for product commercialization. The plant species most often used for manufacturing recombinant antigens are *N. benthamiana* for transient expression; *S. tuberosum*, *N. tabacum*, and *Zea mays* for transgenesis. This can be explained by the fact that *N. benthamiana* and *N. tabacum* are traditional models for these expression systems, whereas *S. tuberosum* and *Zea mays* are easily used as a feed, i.e., for oral delivery of antigen.

In all experiments using the "candidate" plant vaccines, the formation of a specific immune response was demonstrated in vivo, and in most experiments immunogenic proteins were delivered to animals just orally. "Candidate" plant vaccines were usually tested on mice, but in approximately a quarter works the animals subjected to the disease were tested. Protein S of the transmissible swine gastroenteritis virus synthesized in the cells of transgenic maize [55, 62, 63] or tobacco [64] and delivered into the body of pigs as a food supplement, provided for 100% survival of animals after infection [62]. Rabbit protein VP60 virus synthesized in potato [65] and other plants (tobacco, pea, rape) [66] and delivered orally enhanced protective immunity: after infecting rabbits with this virus all animals survived [66]. The effect of plant recombinant antigen was comparable with commercially used vaccines. The use of plant vaccines for the vaccination of wild animals using edible baits (e.g., vaccine against rabies) will lead to an increase in the proportion of wild animal populations having immunity to the rabies virus.

CLINICAL TRIALS AND MARKET LAUNCH OF PLANT VACCINES FOR VETERINARY USE

A potential possibility to reduce the cost of production of biopharmaceuticals using genetically modified plants served at the end of the XX century as an impetus for more than twenty biotechnological companies

to initiate commercial programs. As seen from the Table 1, many biological products of plant origin are developed, expressed in different types of plants and plant cell cultures. For a variety of reasons, including the still skeptical attitude of the human community to the biosafety of genetically modified plants, many of these works remained in the framework of laboratory tests. At the moment three companies function on the biotechnology market of veterinary preparations, two from the United States and one from Canada (Table 2). In the United States the Dow Agro Sciences company presented a recombinant plant viral HN-protein of the Newcastle disease virus (approved by USDA) and a mixture of antiviral vaccines at the first stage of clinical trials. The second American company at Thomas Jefferson University has developed a plant anti-rabies vaccine (completion of phase 1). The Canadian Guardian Biosciences company presented plant vaccine against chicken coccidiosis at the second phase of clinical trials.

Production and wide distribution of biopharmaceuticals is hampered by a number of circumstances. The first of them is related to the problem of biosafety – the cultivation of genetically modified plants in the field can lead to the accidental introduction of foreign genes into crops grown for human consumption. Therefore, most companies producing biopharmaceuticals focused on plant species, which are absent from the food chain of humans and animals and also on growing of genetically modified plants preventing their cross-pollination with other crops. The second difficulty is related to the necessity of plant material treatment for the removal of various undesired compounds, such as lignin, proteases, phenolic compounds, and pigments, especially in the case of plant species, which are not consumed. All these facts result in the requirement of additional studies. The third circumstance is due to the fact that until now all aspects of maintaining and growing of plants producing biopharmaceuticals are not settled at the legislative level. Ambiguity and vagueness of the existing legislation in this area lead to the fact that large biopharmaceutical and biotechnology companies do not tend to invest in the development of technological lines and research

programs in this area, which significantly inhibits the development of the industry.

A significant problem for the development of vaccines for veterinary use, especially those used in agriculture, is the need to minimize the price of the final product. The vaccine should be inexpensive for entrepreneurs engaged in commercial animal breeding and fully subsidized, if you intend to use the vaccine for mass immunization and the prevention of the disease spread in underdeveloped regions. As a result, the potential income of manufacturers of vaccines for animals is much less than that for vaccines intended for humans. For example, in 2007, the volume of the market for the vaccine against human papilloma virus was estimated at more than 1 billion dollars, but the market for the most popular animal vaccines (against foot and mouth disease of cattle and against *Mycoplasma hyopneumoniae* in pigs) together amounted to only 10–20% of this amount [3]. Animal vaccines are cheaper and the volume of market for them is less; therefore, the investments in their development are substantially less as compared with investments in the production of vaccines for humans, whereas the complexity and diversity of both hosts and pathogens in the case of vaccines for animals is much higher.

Expression systems based on the use of plant cells still have a limited application or are used primarily in some laboratories. Nevertheless, biopharming (the biotechnological production of various substances for medicine) in plants has attracted the attention of researchers and manufacturers in developed countries. First biopharmaceuticals of plant origin, such as antibodies (Anti-HBsAg required to purify the hepatitis B vaccine), therapeutic and dietary proteins (“intrinsic factor” required at vitamin B12 deficiency, gastric lipase), have entered the market, that is an excellent illustration of this progress [67].

CONCLUSIONS

Despite the fact that today the number of biopharmaceutical proteins expressed in plant cells is enormous, many questions still remain unresolved. The methods for target recombinant protein quantification and purification are still not developed for most of products. The problems of transgene silencing and increased expression of target protein genes are still at the stage of research. The important task that has yet to be solved is to achieve a stable level of expression in different batches of plant raw material. Not much work appeared for judging about maintaining the stability of recombinant proteins after the harvest, processing, and storage. All of these problems require additional expenses for further research.

One of the main obstacles for the leading research groups working on the development and production of plant vaccines, given the financial constraints, is the fulfillment of the relevant official regulations governing the use of oral medications. To date, the purified

vaccines and therapeutic proteins of plant origin must meet the same standards relating to the production, biosafety, purification, storage, dosage, etc. as any other recombinant proteins for medical purposes.

Nevertheless, despite these difficulties, there were first biopharmaceuticals of plant origin that passed all the necessary tests and were approved for use by the relevant authorities. Some new products having specific advantages over similar products obtained in mammalian cell cultures were developed. Such companies as SemBioSys Genetics Inc. (Calgary, Canada), Medicago Inc. (Quebec, Canada), Protalix Bio-Therapeutics (Karmiel, Israel), and ORF Genetics (Iceland) proved the possibility of quick establishing of the production of purified plant proteins, which are quite competitive in today's market. Progress has been made in the formation of the legal framework related to the cultivation of transgenic plants, testing and use of plant biopharmaceuticals. Several production processes based on transgenic plants have already received a brand GMP (Good Manufacturing Practice), the interest of manufacturers to this field of biotechnology began to increase again.

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