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REVIEWS

Suspension-Cultured Plant Cells as a Platform for Obtaining Recombinant Proteins

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Abstract—Production of recombinant proteins in suspension cultures of genetically modified plant cells is a promising and rapidly developing area of plant biotechnology. In the present review article, advantages related to using plant systems for expression of recombinant proteins are considered. Here, the main focus is covering the literature on optimization of cultivation conditions of suspension-cultured plant cells to obtain a maximal yield of target proteins. In particular, certain examples of successful use of such cells to produce pharmaceuticals were described.

Keywords: transgenic plants, suspension cultures, expression systems, recombinant proteins, biopharmaceuticals

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INTRODUCTION

At present, the market of pharmaceutically valuable proteins is the most developing segment of economics. This is growing faster than the pharmaceutical market in general, and it should reach billion 278.2 US dollars by 2020 according to the forecasts of specialists [1]. More than 200 biopharamaceutical preparations are already represented on the market and more of them are subject to preclinical trials. The largest biotechnological and pharmaceutical companies, such as Epicyte, Ventria, Medicago, Grrenovation, LSBC, and Pfizer, exhibit strong interest in this area and invest in development of scientific studies on creating new platforms for production of pharmaceutical valuable proteins and their industrial application.

To date, most biopharmaceuticals have been produced in cells of mammalians and microorganisms, although both the systems possess a number of drawbacks. Plant cells combine advantages of eukaryotic protein production and certain simplicity and low cost of bacterial. In addition, the use of plants for production of recombinant proteins is an economically significant and promising direction being developed as an alternative for the traditional one. The apparent advantage of plant systems is the lower cost of cell cultivation. These are not subjected to undesirable components, such as bacterial endotoxins, hyperglycosylated proteins produced by yeast, and animal and human pathogens in cell cultures of transgenic animals. Besides, plants are higher eukaryotes, and, therefore, full protein folding and formation of intricate multimeric protein complexes [2, 3] occur in their cells, while a significant part of posttranslational modifications appear to be analogous to those in cells of mammalians [4, 5].

The plant systems being presently developed for recombinant protein expression are extremely diverse and number more than 100 different technologies platforms on different plant species, ways of gene transfer, expression strategies and methods of subsequent target protein extraction, etc. These involve nuclear and plastid transformation, transient [6, 7] and stable expression upon transformation with agrobacterial transfer [8], bombardment or electroporation [9], cultivation of whole land or water plants, and plant tissues or suspension-cultured cells. All these technologies possess certain advantages and drawbacks. Cultures of water plants, such as algae, microalgae, and duckweed, are characterized by high growth rates simply on water or nutrient media simple in composition and sufficiently low in cost, but these require light illumination. Advantages of chloroplast transformation are related to, firstly, high transgene copies' accumulation due to a large number of chloroplasts in each photosynthesizing cell, secondly, the absence of gene silencing effect, and, thirdly, accumulation of target proteins inside the chloroplast capable of protecting the cell against their toxic action [10]. Nevertheless, transplastome systems are perspective ones

only in the case when the target protein is not subjected to intricate posttranslational modifications.

Transient protein expression may be highly efficiently used for the check of created genetic constructions' quality as well as the production of a small amount of proteins during a short time interval [11]. However, drawbacks of the given system are difficulties associated with the observance of proper safety rules in the course of conducting vacuum infiltration into plant leaves of recombinant culture of soil bacterium *Agrobacterium tumefaciens* or plant viruses used as expressional vectors and the necessity of fast isolation of the final product.

Most of the presently existing therapeutic proteins were produced with stable nuclear transformation followed by subsequent isolation and purification of them from transgenic plants–regenerants [12]. As a rule, *A. tumefaciens* is used as a vector, because it is capable of transforming a wide spectrum of dicotyledonous and monocotyledonous plants. Now, in plants of corn, rice, and barley, some technical reagents required for diagnostics are synthesized [13, 14], and there are a number of pharmaceutical products on the stage of clinic examinations [15–17]. Despite evident advantages of using plant systems, the introduction of new methodical designs in industry occurs very slowly, and only a small amount of recombinant proteins produced from whole transgenic plants reached the market. One of the reasons is the negative public opinion prevailing in the area of gene-modified plants as well as imperfection of the legislative framework on whose basis the products in question may reach the consumer.

The present review is devoted to the consideration of possibilities of obtaining recombinant proteins on the basis of expressional plant systems, in particular, suspension cell cultures, and to analysis of the modern state of the biothechnological biopharamaceutical industry.

ADVANTAGES OF SUSPENSION CULTURES' CULTIVATION

A perspective direction to overcome significant drawbacks of using transgenic plants as biofactories may be cultivation of plant cells in fermenters. Although the possibility of obtaining recombinant proteins in plant cell suspensions was demonstrated more than 25 years ago, studies were focused for a long time on the use of whole plants to solve this problem. However, the attitude to using this strategy began to change after commercial surge of interest in obtaining recombinant proteins faced with the absence of regulatory laws and sharp alertness in relation to genetically modified organisms, especially in Europe. Plant cell cultivation technology is known to provide exact observance of cell-growing conditions, unlike field ones, where there are variable weather, climatic, and soil conditions as well as impact of pests, herbivorous animals, and different microorganisms [18]. Growing suspension-cultured cells in sterile reactors not only eliminates the risk of cell culture infection by mycotoxins and pesticides [19, 20] but also lowers to a minimum the possibility of transfer of genetically modified cells into the external medium.

A doubtless advantage of suspension-cultured cell system as compared to growing whole plants is their fast growth as well. Thus, the time required to double cell number constitutes 2–3 days for the exponential growth phase, for example, one cycle of tobacco BY-2 cell cultivation takes approximately 1–2 weeks, while growing whole plants requires several months [21].

A significant advantage of plant cell culture as an expression system is the ability to produce and secrete biologically active proteins through the cell membrane and wall into the intercellular space. This process is metabolically dependent and may be provided by specific leader peptide sequences of both plant and animal origin [22, 23]. Due to accumulation of recombinant protein in the external medium during suspension-cultured cell cultivation, the process of their extraction and purification is significantly simplified. Processing plant tissues and whole plants is associated with a time-consuming and costly extraction procedure, whereas isolation of the target proteins from the medium is simplified in relation to a possibility of fast separation of cells containing a large amount of associated proteins. Target proteins secreted into cultural medium are also characterized by the high degree of their integrity and uniformity, since export of them from cells occurs after their complete processing, i.e., complete removal of signal peptides and attachment of glycan structures if the resulting product is glycoprotein. In the case when recombinant proteins are accumulated in a large amount inside the cells, their obtainment is associated with their purification from nonprocessed, immature, and signal proteins and heterological glycans. According to unpublished data [24], antibodies extracted from cultural medium during growing tobacco BY-2 cells represent a homogenic complex consisting of three glycoforms with dominant one constituting 87%, while the same antibodies from whole plants appear to be represented by six different glycan forms.

Major advantages and drawbacks of suspension-cultured plant cells use are shown in Table 1.

Application of such cells for production of recombinant proteins with different purposes is partly retarded due their accumulation not being high enough, which constitutes, as a rule, not more than 1% of total soluble proteins level [25]. Now, efforts of researchers are focused on overcoming this problem with the selection of optimal conditions of cell cultivation and design of new genetic constructions that are effectively expressed in plant cells and of the methods

Table 1. Advantages and drawbacks of suspension cell cultures as compared to those of other expression systems

Expression system	Plant cell culture	
	advantages	drawbacks
Whole plants	Short cycle of cultivation Simple procedure of separation and purification of product Improving quality of protein product Lower number of legislative and ecological obstacles	Complexities of large scaling the process Higher cost of cultivation Genetic cell instability
Transient expression	Simpler technology of cell transformation Simple procedure of isolation and purification	Longer period of producing the product Lower yield of the product
Cells of mammalians/insects	Lower cost of cultivation Absence of danger of contamination of the product by viruses	Lower yield of the product Specific glycosylation
Yeast	Possibility of the synthesis of complex proteins	Lower yield of the product Lower rate of the growth
E. coli	Correct folding of protein molecules Presence of glycosylation Absence of the danger of contamination by endotoxins	Lower yield of the product Lower rate of growth

to lower the level of target recombinant proteins' degradation.

OPTIMIZATION OF PLANT CELL CULTIVATION CONDITIONS

To enhance plant cell cultures' productivity as to yield of recombinant proteins, cell cultivation conditions are, as a rule, optimized by a number of factors. Here, the main attention of researchers is focused on chemical (nutrient medium composition, organic, mineral, hormonal, pH value, addition of different precursors of target products, etc.) and physical (illumination, temperature, intensity of stirring, etc.) factors.

Nutrient media composition for plant cell cultivation is most often based on certain modifications of the Murashige and Skoog (MS) medium [26], containing all basic mineral and organic nutrient components. Growth of plant cell culture may be enhanced by the addition of small amounts of mineral nutrient components, vitamins, and amino acids, which are precursors of the protein synthesis or growth regulators. Amounts of these substances are varied in dependence on plant species, its genotype, and synthesizing explant possibilities and are often determined in an experimental manner. Changes in medium composition specific for each plant species were designed for rice, soybean, and tobacco cells. Often such modifications influence on cell biomass increase not impacting on recombinant protein production.

Main medium components whose content significantly impact on suspension culture growth intensity are sources of nitrogen, phosphorus, and carbon. Therefore, the selection of their optimal concentrations is of great importance for enhancing cell culture productivity.

It is known that phosphates are the most important nutrient macroelement participating in practically all metabolic processes. Many metabolites have phosphorus in their composition, and these, in particular, are represented by ATP involved in the processes of energy transfer from the photo- and oxidative phosphorylation to energy-dependent cell biosynthesizing ones. Deficit of phosphates is known to be negatively reflected on cell biomass production as a result of the untimely onset of exponential growth phase. Accordingly, it was shown that adequate increase in phosphates concentration is associated with enhancing cell growth and biomass accumulation [27].

Carbon sources belong to a number of the cultivation parameters that are most studied and that significantly impact on biomass formation. As known, sugars are often used as carbon source during plant cells cultivation, and these are represented by glucose, maltose, rafinose or polyatomic alcohols from sugars group, for example, mannitol serving as an osmotic agent that is capable of regulating morphogenesis and cell metabolism [28]. It was shown that sucrose is the most universal carbon source for most cell cultures. Low sugar concentrations in the medium composition lead to cell metabolism rupturing, disturb medium osmotic pressure, and cause cells damage accompa-

nied by releasing proteases disrupting produced recombinant proteins. We need to note that, due to the use of some specific promoters whose action is based on carbohydrate starvation, for example RAmy3D-promoter, the necessity emerged to use alternative carbon sources, such as, for example, fumaric acid, allowing to increase 3.8–4.3 times yield of recombinant protein trypsin in rice suspension-cultured cells [29].

The pivotal factor influencing on recombinant protein productivity in plant cell cultures is the content in the nutrient medium of nitrogen sources and their access. Nitrogen is a macroelement in the medium with the highest concentration as compared to that of other components and it plays a determining role in plant cell metabolism due to its direct participation in the biosynthesis of nuclear acids and protein. The MS medium consists of a mixture of basic nitrogen sources, such as nitrates and ammonium. It was established that the ratio of the latter substances' contents is of great importance for cell cultivation efficiency as compared to that of each carbon source taken alone [30]. In general, it is known that additional nitrogen in the composition of the MS medium enhances recombinant protein production. Thus, in the case of tobacco BY-2 cell cultivation in the medium enriched by nitrogen composition, it was possible to increase 10–20 times production of recombinant antibodies [30, 31].

Solutions of nutrient substances in different concentrations not only determine the nutrient medium value but, as noted above, also impact on cell suspension growth via its osmotic potential. The value of the latter parameter of the cultural medium determined by both its inorganic and organic constitutes contents is known to impact not only on cell lysis intensity but also the rate of cell division in suspension cultures. However, osmotic stress is also used to enhance recombinant proteins yield [32]. This fact is explained by the fact that, for example, mannitol at a concentration of 8% causing some retarding cell growth extends exponential cell culture growth phase during which active cell division and protein synthesis take place, including that of recombinant ones, and, thereby, increase in duration of the synthetic phase of cell cycle leading, in turn, to enhanced accumulation of such proteins [32]. In addition, osmotic stress is known to activate expression of a genes series involved in osmotic response and mitogen-activated protein kinase pathway. Activation of these responses is likely the factor responsible for increased protein expression under osmotic stress.

IMPROVING GENETIC CONSTRUCTIONS

Special attention of researchers and designers of industrial technologies for production of recombinant proteins in plant cells is given to improving the genetic constructions used for cell transformation. Their efforts are directed to enhancing the efficiency of transcription by using more powerful promoters and optimization of mRNA processing and translation.

At present, in plant expressional systems, both constitutive and inducible promoters are employed. Constitutive promoters provide expression of all target genes during all suspension growth phases and simplify industrial preparation, because the reactor can be optimized toward intense cell culture growth. One of most widely used promoters is CaMv 35S, promoter of cauliflower mosaic virus [33]. This allows researchers to obtain, on the one hand, a high level of recombinant protein expression but, on the other hand, may lead, like all virus promoters, to transgene suppression [34]. With constitutive plant promoters, such as "housekeeping" ones, for example, the genes responsible for the synthesis of cytoskeleton components (actin and tubulin), ubiquitin genes, or the genes involved in the protein synthesis of basic metabolic pathways [35], it is possible to minimize similar problems. However, despite all existing varieties of studied constitutive promoters only a small number of them are used for creating the genetic constructions in question.

Although cell cultivation conditions are specially designed to express target proteins at a high level, natural limitations are caused by the very processes of cell division and growth. Besides, at steady synthesis, target proteins may be unstable and susceptible to degradation and inhibition due to their long presence in the cultural medium. For separation in time phases of cell culture growth and recombinant protein production and triggering its synthesis at the moment when the culture achieved optimal biomass, there is a possibility of using inducible promoters initiating the gene expression in response to the action of specific chemical, metabolic, and physical stimulus [36]. In addition, such a strategy is optimal in the case if recombinant protein negatively impacts on cell growth and viability.

In the literature, various inducible promoters employed for obtaining recombinant proteins have been described. For plant cell cultures, chemically inducible promoters are often used, because inductors (alcohols, sugars, etc.) can be purposefully added to the cultural medium [37]. For example, sugar-inducible promoters contain elements responsible for sensitivity of heterologous genes to sugars (SURE). These were found in sporaminic, amylase, and patatine promoters [38, 39] and VvTHI [40]. Using elements of α-amylase promoter makes constitutive rice promoter of actin 1 (actin 1) sensitive to the presence of sugar [41]. The given type promoters activate expression of target genes upon the onset of sugar starvation, i.e., at the time when cell density in suspension achieves the greatest value. At present, αAmy3-promoter is widely used for the production of recombinant proteins in rice cell cultures [42].

A description has been made of a number of genes' functioning in plant defense against biotic factors

whose promoters are induced by specific substances produced by pests and pathogens or formed by the plant itself in response to damage. In corresponding promoters, different combinations of both trans- and cis-activating factors responsible for gene expression are identified. They are revealed in single and multiple copies and different combinations. In different plant species, cis-acting elements are highly conservative and can be used upon creating pathogen-induced transgenic systems [43, 44]. These elements were shown to function in different nucleotide context of promoters. For each element, the number of copies influences on the force of promoter induction that makes their application suitable upon using pathogen-induced gene expression [45].

Promoters induced by increased temperatures activate so-called *HSP*-genes responsible for the synthesis of stabilizing proteins (heat-shock proteins) and other cell components providing plant thermotolerance. These promoters are also highly conservative in different plant species [46]; they may be universal or tissue-specific [47] and activate gene expression at temperatures above 25°C. Advantage of such promoters is in the absence of any intervention in chemical composition of the medium and in lowering risk of additional contamination of the cell culture.

Processing mRNA, i.e., capping, splicing, and polyadenylation, may also impact on target protein production. The sequences located directly behind stop-codons are pivotal for processing, contain polyadenilating signals, and significantly affect gene expression level in cells [48].

We need to note that the role of introns in eukaryotic gene expression was finally not elucidated. Nevertheless, it was reported that involvement of some of them in transgenes exert an enhancing effect on gene expression. There are several plant introns described in the literature that were introduced into the composition of heterological transgenes in the form of synthetic introns. These are represented by intron 1 of genes encoding rice actin, maize ubiquitin, alcohol dehydrogenase, and some other proteins [35]. As noted here, the given sequences are capable of stimulating transgene expression as compared to its natural analogues.

The stability of transcript in the cytosol has no less important value. There are specific destabilizing sites, so-called transcript-terminating sequences, whose presence may lead to lifetime shorting of mRNA molecule. This problem is of special actuality in the case of gene expression in mammalians, where the presence of similar sites may be a significant factor lowering the yield of target product [49]. Such sequences must be identified and modified or removed from the gene sequence.

Efficiency of recombinant protein synthesis at the translation stage can also be enhanced with the gene-engineering techniques. It is believed that efficiency of the translation depends on the rate of the initiation which in eukaryotes is provided by interaction of ribosome subunit 40S and capped 5'-end of mRNA followed by passing through nontranslating leader sequences to the first AUG-codon, which is the initiating one for 92% of studied plant genes. The nucleotide context and structure of leader sequences are capable of affecting all steps of this process. In plants, consensus sequences around initiating codon AUG are distinguished from those in analogous systems of animals. Their optimization for the plant systems leads to higher efficiency of the translation.

Optimization of nucleotide sequences of the encoding part of the gene as applied to the system where its expression will occur has an analogous action. It is known that there are codons that are predominantly recognized by tRNA and rarer ones recognized by tRNA but with lower efficiency. "Preferable" codons are distinguished in animals and plants, dicotyledonous and monocotyledonous, in nuclear and plastid genome of the same plants. Using the strategy based on optimization of codon composition, researchers were able to achieve 100-fold enhancing expression of gene *crylA Bacillus thuringiensis* in transgenic tobacco and tomato and in the experiments with gene *gfp* in tobacco cells [50].

DEGRADATION AND ADSORPTION OF RECOMBINANT PROTEIN

Low yield of recombinant proteins produced in plant cell culture is explained not only by their low expression in the plant system but to a considerable extent by degradation level of the final product. It is known that disruption of recombinant protein occurs under the action of proteolytic enzymes [51, 52]. This process most often takes place in the cytosol and, therefore, its intensity can be significantly lowered upon accumulation of target proteins in intracellular compartments, such as EPR, due to attaching to target protein of the signal peptide (for example, KDEL and HDEL) responsible for anchoring the final product in it. In addition, EPR contains a large amount of molecular chaperones involved in the protein folding and formation of intricate protein structures. There are reports on manifold, one and two orders of magnitude, increase in the yield of recombinant protein only thanks to its compartmentalization in EPR. One of the most dramatic examples is 10000-fold increase in the yield of epidermal human growth factor (EGF) in tobacco plant cells when it is accumulated in this organelle [53]. However, this strategy is not always applied, because, in some cases, the most important steps of posttranslational protein processing and its transformation into the functional form occur in the Golgi apparatus [54].

To lower the level of the proteolitic degradation, other strategies have been designed as well. They involve coexpression of recombinant proteins and

protease inhibitors [55], obtaining the knockout mutations of genes for specific proteases [56], and removing from target proteins the protease-binding sites, as well as RNA interference, to suppress proteases synthesis [57]. The above strategies allow researchers to enhance target protein production more than two times. However, the given methods were not completely designed and require further study.

It was shown that considerable losses of the proteins in question occur in the case of its export in the cultural medium [58]. Such secreted proteins may exhibit instability or degrade under the action of proteolitic enzymes that are released in the medium in the course of cellular vital functions. However, according to the data of Doran [59], upon addition, for example, of immunoglobulin IgG1 to the sterile medium for cell cultivation at complete absence in its composition of proteases and plant biomass full protein degradation was observed for the first 2 hours. At the same time, addition to the above medium of stabilizing agents, such as polyvinylpyrrolidone (PVP), considerably increased durability in it of antibodies [51]. Their stability was also increased in the medium obtained after cultivation in it of suspension tobacco cells or hairy root culture. These results are putatively explained by the protective effect of polysaccarides and proteins exceeding the action of extracellular proteases [60].

At present, there are the methods designed and still undergoing improvement that are capable of increasing the yield of target proteins by using low molecular weight proteins (gelatin, BSA, etc.), protease inhibitors or protein-stabilizing polymers (PVP, polyethylene glycol, PluronicF-68, etc.) in the composition of the cultural media [61], and also with adjusting its osmotic pressure to minimize cell lysis leading to proteases releasing [62]. The above stabilizing agents are characterized by the absence of phytotoxicity, at least, at usable concentrations, and do not influence on cell viability and their growth and capability for division.

One more problem associated with the production of recombinant proteins is in the ability of proteins to adsorb on any surface independent of whether it is hydrophobic or hydrophilic or it is charged positively or negatively [63]. The protein-surface interactions have been intensively studied for the last 10–15 years not only in connection with the proteins production but also with the purpose to lower the protein absorption in the course of their membrane filtration, blood and plasma maintenance, and also upon using different devices and implants in medicine. It was shown that protein adsorption leads not only to lowering its amount in the medium but also changing its conformation, and, therefore, ultimately its denaturation and inactivation. Losses of protein as a result of its absorption are the more significant the greater the size of its molecule. This is explained by greater force of the interaction of large molecules with surfaces and the presence of higher amount of binding sites. In studies performed by Nakanishi et al. [63] and Imamura et al. [64], the interaction of proteins with different type surfaces of the equipment usually used for cell cultivation and also isolation and purification of the final product were studied. It was shown that the highest level of their absorption is in glass vessels, while the lowest is in plastic vessels. Using BSA and gelatin as a special coating considerably increases the yield of recombinant proteins as well, with due to not only decreased adsorption of them but also lowering their degradation due to proteolysis. However, employment of such stabilizing agents is limited in connection with the danger of contamination of cell cultures by the virulent factors of animal origin.

CELL SELECTION AND SCREENING

It is known that plant cell cultivation in vitro causes elevation in the level of genetic variability, and its extent depends on the duration of this process. Young cultures during the first months of their cultivation exhibit, as a rule, elevation in their heterogeneity. In contrast, the genome stabilization with the maintenance of certain ploidy level and slowing the rate of mutation is inherent in long-cultivated cells [65]. Moreover, after genetic transformation, suspension cell culture exists in the form of a mixture of transgenic and nontransgenic cells. Thus, the task of researchers is the design of effective selection methods and the maintenance of maximally stable culture of transformed cells. The selection of transgenic cells is usually carried out due to introduction of the genes tolerant to antibiotics and herbicides into genetic constructions that confers transgenic cells the ability to survive and be divided in the medium containing selective agents.

An interesting alternative method of transgenic plant cells selection is one based on the principle of "positive selection." This is in using cytokinin derivative, glucuronide, as a selective agent and gene β-glucuronidase (*gus*) of *E. coli* as a selective gene. Only the transformed cells carrying gene *gus* are capable of splitting glucuronide of cytokinin to its active form and growing in the medium not containing cytokinin. Commercial realization of the given method is presently retarded only by high cost of the usable selective agent [66].

An analogous method of selection is based on the use of phosphomannose isomerase (PMI) as a selective marker [67]. Although plant cells are not properly capable of metabolizing mannose transgenic cells, they can transform mannose into fructose with gene PMI and use it as a carbon source. In contrast, nontransgenic cells die as a result of carbon starvation.

After transformation, plant cell culture represents a heterogenic population of genetically and epigenetically different cells that are distinguished in their expression level, the amount of transgens copies, and the insertion sites, which considerably influence on

their productivity. Only a small portion of cells from the initial transformants produces recombinant proteins with high efficiency, and, therefore, selection of "elite" highly productive cells and then use of them for creating monoclonal cell suspension cultures is of great importance. However, particularly such cells often grow less actively and may be supplanted by their low productive cell environment. Fast selection of enough productive cells can be achieved by using, for example, the technique of fluorescent-activated cell sorting (FACS). For this purpose, cells must express the fluorescent marker fused with the target product and, thus, labeling transgenic cells. The single cells characterized by most intensive fluorescence selected as a result of sorting are placed into minichambers together with nontransgenic cells in order to achieve a certain cell suspension density required for its optimal growth. Subsequent application of the factor selective against nontransgenic cells allows researchers to obtain monoclonal cell suspension with high productivity of recombinant protein [68].

In recent years, new methods for directed editing of genomes have been designed. In particular, these are site-specific plant cell transformation, such as RMCE (recombinase-mediated cassette exchange) and ZFN (zinc finger nuclease), and also technologies of using TALEN and CRISPR/Cas [69]. To date, editing plant genomes with the TALEN system was performed on four model objects [70]. Thanks to the given technologies, there is the possibility of transgenes' insertion into expression-active sites of the plant genome that provides stably high production of recombinant proteins.

Additional difficulties may emerge during long plant cell cultivation and periodic subcultivation, and these may be caused by gene silencing or recombinant events leading to lowering or even complete disappearance of expected protein production. Although differentiated plant cells are morphologically stable, they are lacking fully functional plasmodesmata and possess reduced system of posttranscriptional gene silencing (PTGS), i.e., are characterized by genetic instability. Possible reasons of such instability are the somaclonal variability, loss of transgenes, or transcriptional gene silencing. Epigenetic silencing is believed to be a major factor responsible for transgene instability in long-lasting plant cell culture. Due to genetic instability, the cells can lose the capability of high productivity of recombinant protein, and, as a result, the cells with low productivity will be predominant ones in the cell culture. It was shown that production of IgG1 in tobacco cell culture remained constant for not more than 3 years or, according to other data, during 250 passages [71]. Therefore, technology of maintaining elite cell lines in so-called cell banks is being developed at present. These banks are characterized by the presence of a three-step system consisting of a cell bank, a master-cells bank, and a working cell bank. One of the possibilities for preserving elite cell lines is their cryopreservation. An alternative technique is continuous screening of highly productive cell lines supporting the maintenance in the culture of a high expression level of recombinant protein, including a creating mathematic models characterizing the stability of the production and providing planning of the works. In addition, the techniques of coexpression of gene silencing suppressors capable of maintaining high productivity of elite cell lines are being designed [36].

SUSPENSIONS OF TRANSPLASTOMIC CELLS

It was suggested earlier that one of the technologies for recombinant protein production may be the cultivation of suspensions of transplastomic cells. Technology of transforming chloroplasts was designed as a highly promising technique for obtaining recombinant proteins. During plastid transformation, there are no problems characteristic for nuclear transformation, such as gene silencing, epigenetic effects, or variability of transgenes expression. A high expression level of genes transferring into the chloroplast genome or plastome is largely determined by a high copies number of plastid DNA. For example, mesophyll cells of *A. thaliana* contain approximately 120 chloroplasts that constitutes approximately 1000–1700 copies of 154000 nucleotide pairs (n.p.) of the plastid genome, while leaves cells of *Nicotiana tabacum* contain 10000 copies of 156000 n.p. of the plasid genome. Numerous studies showed that chloroplasts have great possibilities for expression of foreign proteins at the level achieving 70% of total soluble protein [72, 73] and significantly exceeding that upon the nuclear transformation. However, during study of the expression level of recombinant proteins in transplastomic leaf tissue, callus or cell suspension using as examples the protein GFP—easy visualized in plant cells by fluorescence microscopy [74]—and protein TetC, which is fragment C of toxic causative agent of tetanus and usable as a component of edible vaccine [75], it was shown that their expression in suspension cells is albeit high enough but yet reliably lower than that in leaf tissues of transplastomic plants. This fact motivated researchers to create a new type of bioreactors of transient immersion for the induction and leaf tissue cultivation with high production of recombinant proteins being initiated by transplastomic cell suspensions [76]. Possible reasons of higher accumulation of such proteins in the biomass being cultivated in the above bioreactors are a different number of plastids per cell, a disturbance in chloroplast development in long-lasting cultivation of cell suspensions, and differences in the activity of promoters. Thus, the activity of light-regulated promoter PsbA used in the studies in question was declined in suspension culture leading to lowering protein expression. The authors believe that it is of interest to compare the expression levels in photoautotrophic suspension cultures with those in classic plant cell cultures.

Table 2. Some biopharmaceutical proteins synthesized in plant cells

Product	Plant species	Reference
Antibodies		
Immunoglobulins sIgA, IgG4 and IgG1	*N. tabacum* cv. BY-2	[84, 54]
Against surface hepatitis virus antigen B (HBsAg mAb)	*N. tabacum* cv.BY-2	[85]
Against rabies virus	*N. tabacum* cv. Xanthi	[86]
Against surface HIV antigen (2G12)	*N. tabacum* cv. BY-2	[87]
Therapeutic vaccines		
Human transglytaminase (htTG)	*N. tabacum* cv. BY-2	[89]
Lysozyme	*O. sativa*	[90]
Glycocerebrosidase	*D. carota*	[91]
DNAase I (DNase I)	*D. carota*	[96]
Hormones and cytokines		
Interleukines IL2, 4, 12, 18	*O. sativa, N. tabacum* cv. BY-2	[93, 94, 96]
Epidermal human growth factor (hEGF)	*O. sativa, N. tabacum* cv. BY-2	[53, 96]
Insulin-like factor-1 (IGF-1)	*N. tabacum* cv. By-2	[96]
Granulocyte macrophage colony-stimulating human factor (hGM-CSF)	*O. sativa*	[96]
Vaccines		
Hemagglutinin-neuraminidases (HN)	*N. tabacum* cv. BY-2	[98]
Hemagglutinin (NA)	*N. tabacum* cv. BY-2	[98]
E. coli thermolabile toxin	*N. tabacum* cv. BY-2	[98]
Other proteins		
α_1-antitripsin	*O. sativa*	[41]
Lactoferrin	*Acanthopanax senticosus*	[41]
Briodin-1	*N. tabacum* cv. BY-2	[96]

Creating photoautotrophic suspension cell cultures might have additional advantages. However, most cultivated cell suspensions require the presence of sugar in the medium composition and are characterized by lowered photosynthetic activity or even its absence. Therefore, solving this problem is a large enough difficulty. There are only a small amount of plant species, for example, *Chenopodium rubrum* and *A. thaliana* [77], for which such cultures were produced. They are employed exclusively for studies of different aspects of photosynthesis, formation of secondary metabolites, etc.

ADVANCES OF PLANT BIOTECHNOLOGY

Plant biotechnology is presently an intensively developing area, and production of pharmaceutically valuable recombinant proteins in the plant cell system may be a promising alternative for their production in a whole plant [78]. Here, cell lines of tobacco BY-2 (Bright yellow-2) and NT-1 (Nicotiana tabacum-1) and also cells of rice [79], soybean [80], and tomato [81] are the most suitable for this purpose. These cell cultures are characterized by high growth rate, synchronization of cell cycle, and susceptibility of transformation with *A. tumefaciens*. At present, the production in the plant systems of antibodies, vaccines, hormones, plant growth regulators, cytokines, therapeutic enzymes, etc. is being actively developed (Table 2).

Antibodies are the most important class of biopharmaceuticals [82]. They are relatively stable and may be accumulated in high concentrations (to 100 mg/L). These proteins can be obtained from the medium or extracted with affinity chromatography, and their biological activity is easily tested. Thus, recombinant antibodies are an ideal example for demonstration of advantages of the plant systems for production of recombinant biopharmaceuticals.

With the production of the first full-sized monoclonal antibodies 20 years ago [83], interest in their production in plant cells was not diminished. In particular, already a number of functional immunoglobulins G (first of all IgG1) and immunoglobulin A have been produced [84]. For some complex molecular forms of immunoglobulins, such as secretory IgA (sIgA) and IgG4, plants are the only system commercially realized [54]. Interest in production of monoclonal antibodies in plant systems is now growing with increasing concern for safety problems, for example

upon producing them against the surface antigen of hepatitis B virus [85], human monoclonal antibodies against rabies virus [86], and human immunodeficit virus [87]. In addition to full-sized monoclonal antibodies in plants and plant cells, some other antibodies used in therapeutic purposes, such as Fab-fragments, single-chained Fvs (scFv), bispecific Fvs, inclusion bodies, and single-domain antibodies, can be successfully synthesized [54]. In our country, the expression of genes for recombinant minimal antibodies (miniantibodies) against human spleen ferritin in the form of the protein fused with barstar was realized in suspension culture *N. tabacum* [88].

Some enzymes of therapeutic or diagnostic purposes are produced in the plant systems as well. For example, the possibility of producing human tissue transglutaminase (htTG), the enzyme being used for diagnostics of celiac disease, was demonstrated [89]. In rice cells, human lysozyme was synthesized for potential use as an antimicrobial food additive [90]. Some human lysosomal enzymes were produced in BY-2 cells, including α-iduronidase employed in replacement therapy of mucopolysaccharidosis I. For human, the first medicine preparation became glucocerebrosidase (α-talylglucoceferase) synthesized in genetically modified carrot cells by the Protalix [91]. The largest pharmaceutical company Pfizer (United States) became the first pharmaceutical company that reported on the introduction into the market of a preparation synthesized by plant cells in bioreactors. The company acquired rights to production of enzyme α-talyglucoceferase from Protalix. According to a company representative [92], Pfizer has determined a strategy on employment of the innovation platform for the biosynthesis of the preparations in plant cell culture. This is an important step towards cost lowering and elevating the efficiency of biopharmaceutical drug production. A biological analogue of an inhibitor of tumor necrosis factor alpha (etanercept) and a candidate for replacement enzyme therapy of Fabri disease have already entered the preclinical level. The company has interest in using this platform for the synthesis of therapeutic agents for treatment of rare genetic diseases as well as other medicine preparations.

Ways for obtaining different cytokines, growth factors, and hormones in tomato, tobacco, rice, and potato cells have been designed. In tobacco cells, biologically active human interleukins 2, 4, 18, and 12 and human granulocyte-macrophage colony-stimulating factor were synthesized [93, 94]. Human growth hormone was synthesized with its high content in rice (57 mg/L) and tobacco (35 mg/L) cells [55, 95]. A possibility for producing some other growth factors in plant cells, for example epidermal growth factor (hEGF) or insulin-like growth factor (IGF-1), was demonstrated, although their production is carried out at a low level so far [53, 96, 97].

In plant cell cultures, in particular in BY-2 cells, some pharmaceutical proteins for diagnostics and immunotherapy are synthesized, for example, allergen of dust mite; in rice cells, α_1-antitripsin; and human lactoferrin in rice and carrot cells [41].

Plant systems for production of subunit vaccines are of especial importance, and these, unlike traditional vaccines, are represented by only selected immunogenic epitopes of an infectious agent capable of causing immune response in an organism. A unique feature of vaccines produced in the plant systems is the fact that plant cells can simultaneously serve as a means of delivery of oral vaccines.

The first vaccine for veterinary obtained in the plant system was one against the Newcastle disease of birds produced in tobacco NT-1 cell culture in the Center of Veterinary Biology in the United States (USDA Center for Veterinary Biologists) in 2006 [98]. Numerous antigens were expressed on the basis of Concert™ Plant-Cell-Produced System platform designed by Dow AgroSciences, for example, thermolabile toxin *E. coli*, hemagglutinin of neuraminidase (NM), hemagglutinin of bird flu, and VP2 of infective bursitis (IBD). These vaccines produced in the plant systems exhibited effective immune response in birds and pigs and provided a high extent of defense of animals against pathogens [98].

Soon the producing recombinant protein in suspension plant cell cultures will certainly become the most often used platform of all plant systems being presently employed. The initially dominant idea of using whole plants for the same purpose was completely revised after the initiation of production of α-talygluceferase in carrot cells for application of it by adults in 2012 and in pediatrics in 2014 [91]. This advance has opened a pathway to complete adoption of this technology. At present, several new products are under clinical trials and, as expected, will soon be introduced into the market. One more factor contributing to the progress of plant cells in production of biopharmaceuticals is their complete accordance to principles of GMP (Good Manufacturing Practice or Proper Production Practice) as compared to whole plants and also society's preferences of biopreparations being produced in cultivated cells as compared to genetically modified plants.

Production of recombinant proteins in the plant systems is again retarded by the necessity of solving one of the most important problems: low productivity as compared to that of mammalians' cell cultures. However, this problem exists mainly due to the later emerging of plant cells as the competitive platform and, accordingly, lower investment and experience towards the optimization of plant cell cultivation processes as compared to those of mammalian cells. In this connection, significant progress in developing production and processing of recombinant proteins is soon expected, and this involves optimization of the

nutrient media, engineering processes, and bioinformational and statistical planning of experiments.

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