Transplastomic Plants: Problems of Production and Their Solution

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Abstract—The major problem associated with production of transgenic proteins in plant expression systems is the low level of their accumulation. Transplastomic plants with a transgene incorporated not in the nuclear but in the plastid genome (plastome) in some cases ensure elevation of the yield of the desired protein dozens of times and more. However, two problems arise in this case: low frequency of integration of transgenes into the plastid genome and difficulties with production of homoplastomic and homoplastidic plants. Recently, a number of tendencies to overcome these difficulties have been outlined. The first problem is being solved via an improvement of the systems of delivery of exogenous DNA and a fine design of the vectors and the second by multistep screening on several antibiotics and the use of dedifferentiated tissues for transformation. This review deals with a detailed analysis of these problems and their solution.

Keywords: homoplastidity, homoplastomy, transplastomic plants, expression of transgenes, efficiency of plastid transformation

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INTRODUCTION

Plastids whose most known representatives are chloroplasts are independently dividing semiautonomous organelles of a plant cell. Apart from chloroplasts, there are various types of plastids, such as proplastids, etioplasts, amyloplasts, and chromoplasts [1, 2]. They perform not only photosynthetic but also other important functions in the plant cell, such as synthesis of fatty acids, amino acids, vitamins, pigments, and many other compounds [3, 4].

Plastids possess their own genome (plastome) exposed to genetic manipulations. The plastome composed of circular DNA is usually rather small (~150 kbp) and contains approximately 120 genes. Each plastid bears approximately 100 copies of the plastome. The plastome’s ability to independently replicate in the cell makes plastids a unique and highly efficient biological platform. The structure of the plastid genome, the organization of genes as operons, and the presence of bacterial-type RNA polymerase and 70S ribosome corroborate the plastid origin from ancient free-living cyanobacteria. Plastids are also capable of posttranscriptional processing mRNA [5]. Gene expression in plastids is regulated both at transcriptional and posttranscriptional levels, including stabilization of mRNA and the effect of various translation factors [6, 7].

Recently, plastid transformation has become very attractive for biotechnologists since it has a number of advantages over nuclear transformation. (1) When traditional methods of transformation are used, the transgene is incorporated into a random region of the nuclear genome by means of the NHEJ mechanism, whereas incorporation in plastids (as prokaryotic structures) predominantly obeys the mechanism of homologous recombination (HR). This makes it possible to precisely insert the transgene into a specified region of the plastome and avoid the position effect and overlapping of it with plastome own genes. (2) Since plastid genes are transcribed as operons, it becomes possible to together insert several genes of foreign metabolic pathways and create artificial operons in plastids. (3) Transplastomic plants contain not merely one or two copies of the transgene per cell as usually occurs in the case of nuclear transgenesis but by copy in each of ~100 elementary plastomes in all of ~100 cell chloroplasts, i.e., up to 10000 copies [8]. In combination with the other two characteristic properties of plastids—the inherent ability to express large quantities of gene products and lack of gene silencing and epigenetic modifications [9]—the quantity of accumulated transgenic protein expressed in plastids may be 10–100 times larger than in the case of nuclear expression [10, 11] and may come to 70% of the total soluble pro-
tein of a plant without detriment to its viability [12]. Another important feature of plastids is their inheritance along the maternal line. This makes it possible to maintain a stable expression of a transgene even in the case of cross-pollination and rules out transfer of recombinant genes with pollen to other plants. Finally, each plastid is an isolated compartment. Although many metabolites may partially get to the cytosol most of them and the genetic material are protected by plastid double membrane and isolated from catabolic enzymes of the cytoplasm. Some vaccines produced in plastids of *Chlamydomonas reinhardtii* and stored as freeze-dried cells at room temperature were shown to be stable and preserved activity for at least 0.5 and 1.5 years [13, 14].

Pioneering transformations of plastids were performed in unicellular alga *Ch. reinhardtii* and in tobacco plants more than 25 years ago [15, 16]. Subsequently, the list of plant species with transformed plastids increased slowly and with great difficulty. However, transplastomic plants were produced in tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), lettuce (*Lactuca sativa*), soybean (*Glycine max*), cultivated cabbage (*Brassica oleracea var. capitata*), cauliflower (*Brassica oleracea var. botrytis*), sugar beet (*Beta vulgaris*), egg-plant (*Solanum melongena*), carrot (*Daucus carota*), cotton plant (*Gossypium hirsutum*), and poplar (*Populus alba*) [17, 18]. Not long ago, a great success was achieved in the production of fertile transplastomic plants of *Arabidopsis thaliana* [19]. Transplastomic plants currently produce a wide range of proteins designed for different purposes. On a larger or smaller scale, chloroplasts produce dozens of various proteins: antigens, recombinant vaccines, monoclonal antibodies, and enzymes for medicinal and commercial application [20–23].

Thus, transplastomic plants promise much in terms of industrial production of a whole line of biological preparations. However, there are some problems restricting the use of transplastomic plants as bioreactors. These problems have two main aspects: (1) low probability of plastidal genome transformation and (2) difficult selection of material for production of homoplastomic and homoplastidic plants. The first problem implies engineering of particular species-specific recombination cassettes. Moreover, the efficiency of transformation depends a lot on the modes of delivery of these cassettes to a plant cell. At the same time, intensification of target protein biosynthesis depends on a multistage screening on several antibiotics and elaboration of special programs of selection for attaining homoplastomy and homoplastidity of plant cells. This review deals with these issues.

**WAYS OF ELEVATING PLASTIDAL GENOME TRANSFORMATION FREQUENCY**

Elevating the frequency of target genes’ integration into plastids remains one of the most serious problems associated with the use of plastids in biotechnology. This task is complex and carried out at different levels. They include the methods of delivery of exogenous DNA to plastids, design of species-specific expression cassettes (optimal promoters and 5’- and 3’-regulatory UTRs), choice of the site of integration into the plastome, and use of tools of target genome editing.

**Delivery methods of foreign DNA to the plastid genome.** Most often, foreign DNA is delivered to plastids by the biolistic protocol, i.e., by means of gold or tungsten microparticles [24] and transfection in the presence of polyethylene glycol (PEG). Unfortunately, the latter method requires protoplasts, i.e., removal of the cell wall [25]. Biolistic delivery may be used for the cells of essentially all plant species since it allows the researchers to modify a lot of gene gun parameters: distance to the target, pressure in vacuum and blasting chambers, size of microparticles, and DNA concentration. This makes it possible to deal with any structure of the plant cell envelope. Culturing of protoplasts in the presence of PEG vesicles coated with plasmid DNA leads to uptake of this DNA by the protoplasts with its subsequent integration into the plastid genome [25]. On the one hand, the PEG method is more labor-consuming as it requires lysis of cell walls and production of protoplasts; on the other hand, it is cheap since it does not require expensive equipment.

A novel strategy of plastid transformation by means of nanoparticles has recently been developed; it does not require the production of protoplasts or use of a gene gun [26]. This method makes it possible to deliver DNA to chloroplasts of leaf cells by means of single-walled carbon nanotubes (SWNTs). These nanocarriers are carbon nanotubes associated with chitosan (CS-SWNTs). They bear a positive charge and can bind negatively charged plasmid DNA owing to electrostatic interactions. Upon infiltration into plant leaves, such a DNA-SWNT conjugate can easily cross the cell wall and get to the mesophyll cells; then, in accordance with the mechanism of lipid exchange envelope penetration (LEEP), it can easily pass through the chloroplast lipid double membrane [26]. This strategy of delivery to chloroplasts is based on the fact that DNA can be selectively liberated from the DNA-SWNT complex within the chloroplast owing to the pH difference between cytosol and chloroplast medium. Weakly acid cytosol of mesophyll cells ensures the retention of DNA in the chitosan complex, whereas DNA is easily released in weakly alkaline chloroplast. This results in a considerable accumulation of DNA in chloroplasts. In fact, up to 35% of SWNT migrate to chloroplasts and up to 88% of chloroplasts contain SWNT [26]. These figures are much higher than those obtained upon biolistic or PEG-mediated transformation of chloroplasts. Moreover, the efficiency of DNA delivery to chloroplasts depends for the most part on dimensions and hydrodynamic properties of SWNT and not on DNA concentration.
In their experiments, the researchers used much less DNA (1000 times less that in the case of PEG-mediated transformation and 250 times less than upon biolistics) and yellow fluorescence protein gene YFP as a reporter. Intense fluorescence associated with chloroplasts was observed when the ratio between DNA and SWNT was 1:6 and vanished at 1:1. Excess DNA bound with SWNTs apparently neutralizes their surface charge; as a result, they lose their ability to pass through membranes [26]. The authors have shown the efficiency of the SWNT method of DNA delivery to chloroplasts in four plant species belonging to different families, which points to a great potential of this method for its wide application [26]. Unfortunately, these experiments used only one plasmid with flanking sequences from plastome of *Panicum virgatum*, but this species was not included in the investigation. Therefore, there is no evidence that the reporter gene was integrated into the plastome, and the researchers studied only its transient expression. Thus, the integration of target genes into the plastome by means of SWNT is to be shown.

Another efficient method of delivery of genetic material to plastids by means of leaf infiltration has recently been worked out. The method is based on the formation of a complex of plasmid DNA (pDNA) with two signal peptides comprising 30–35 amino acids [27]. Peptide BP100 ensures passage through the cell wall and cytoplasmic membrane (cell-penetrating peptide, CPP); the second peptide CTPK9OEP34 is responsible for transport via the intracellular membrane and cytoskeletal networks to chloroplasts (cytoplasmic transduction peptide, CTP). These two peptides electrostatically interact with plasmid DNA and produce a globule 170–200 nm in size. In 2 h after infiltration, pDNA/CPP/CTP complexes bind with chloroplast membranes, then penetrate therein, and release pDNA. In 24 h as a result of transient expression of reporter genes, their products (GFP, RedFP, and luciferase) are detected in the chloroplasts. The authors compared efficiency of biolistic delivery of genetic material to chloroplasts and delivery by means of pDNA/CPP/CTP complexes. Biolistics brought about emergence in the cell of not many transformed chloroplasts, whereas the use of pDNA/CPP/CTP complexes caused transformation of 10–12 chloroplasts per cell. The activity of luciferase in transformed chloroplasts produced by the latter method was three to ten times higher than in the case of biolistics [27]. The same as in the method described above, the researchers sadly investigated only transient expression of reporter genes.

**Engineering of expression cassettes.** In contrast to the nuclear genome where homologous recombination (HR) is difficult, incorporation of expression cassettes into the plastid genome predominantly obeys the HR mechanism, which ensures its precise insertion into a predetermined locus of the plastome [8, 28]. Frequency of homologous recombination is the greatest when the cassette is flanked on each side by sequences of not less than 121 bp homologous to the sequences at the integration site [29]. Elevation of the frequency of integration and intensity of transgene expression depend a lot on the site of insertion and correct choice of the elements of the expression cassette: the promoter and 5’- and 3’-UTRs. The most frequently used combinations of promoters and these untranslated regulatory elements ensuring the most active expression of transgenes are given in Table 1.

**Use of the CRISPR/Cas9 system of genome editing.** Efficient incorporation of the expression cassette into the plastome controlled by the HR mechanism depends heavily on the presence of a double-stranded break (DSB) of DNA in this region of the plastome [31], which may be induced by the CRISPR/Cas9 system of genome editing. Precise positioning of DSB is ensured by sgRNA bearing a sequence homologous to the target locus and directing endonuclease Cas9 thereto. Yoo et al. [32] used the CRISPR/Cas9 system for target integration of reporter gene GFP into the plastome of *Ch. reinhardtii*. The researchers have engineered a package plasmid containing three expression cassettes: the first with gene Cas9, the second with sgRNA, and the third with a donor cassette with reporter gene GFP and the genes of selectable markers. The donor cassette was flanked by sequences homologous to the regions of the integration site necessary for its insertion in accordance with the HR mechanism.

| Table 1. Promoters, regulatory untranslated regions (UTRs), and insertion sites commonly used for plastid transformation (quoted from [30]) |
|---|---|---|---|
| Promoters | 5′-UTR | 3′-UTR | Insertion sites |
| *psbA* | *gaggg* | *psbA* | *trnA1* |
| *rnn* | *T7g10* | *rps16* | *rbcL* |
| *rbcL* | *rbcL* | *petD* | *rrn* |
| *psbA* | *petD* | *trnfM-trnG* | |
| *atpB* | *psbA* | *trnN-trnR* | |

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and the sites of Cas9/sgRNA recognition, which ensured its excision from plasmid. The plasmid was delivered to the chloroplasts by the biolistic technique. As a result of this experiment, two of 20 produced transformants contained the reporter gene at the pre-determined locus of the plastome. It is interesting that no target transformations were obtained when the plasmid did not contain gene Cas9, which suggests that DSB is necessary for incorporation mediated by the HR mechanism [32]. An identical genetically engineered construct bearing the system of genome editing was used for transformation of tobacco chloroplasts. The authors showed that target DSBs induced by the Cas9/sgRNA complex elevate the frequency of chloroplast transformation by six to ten times [33].

**Effect of species-specificity of sequences and choice of the site of integration into the plastome on the frequency of insertion and expression of the transgene.** Coding regions in the plastid genome are rather conserved; at the same time, intergenic spacers into which genes are integrated may considerably differ in different species. For instance, only four of almost 150 intergenic spacers are conserved in dicotyledons [34], and monocots have no conserved spacers [35]. Even one nucleotide substitution in the exogenous flanking sequence of the expression cassette may bring about a dramatic reduction in the frequency of integration mediated by the HR mechanism. Within a species, intergenic spacers are usually identical [34]. Therefore, each species requires a specific vector with flanking sequences totally homologous to the intergenic spacer.

In the chloroplast genome, the customary position effect is not observed; however, there are intergenic spacers with a high or low transcription activity. Therefore, a wise choice of an integration locus is very important for ensuring a high level of transgene expression. The best sites used for plastid transformation were \( \text{rrn}12/\text{trnI} \) (tomato), \( \text{rrn}26/\text{trnG} \) (cauliflower, potato, and lettuce), \( \text{trnL}/\text{trnM} \) (carrot and lettuce), and \( \text{trnV}/\text{trnI} /\text{trnA} \) (soybean). However, the highest level of transgene expression was obtained upon integration into transcriptionally active spacer \( \text{trnL}/\text{trnA} \). The overwhelming majority of all the produced transplastomic plants were obtained via integration into particularly spacer \( \text{trnL}/\text{trnA} \), which suggests that it has a great potential for ensuring high levels of transgenes’ expression [36].

**Promoters.** Plastids of higher plants contain RNA polymerase of two types. One of them (PEP, plastid-encoded RNA polymerase) is encoded in the plastome and was passed to them by a cyanobacterial endosymbiont. Polymerases of the second type (NEPs, nuclear-encoded RNA polymerases) are encoded in the nuclear genome; they closely resemble polymerases of bacteriophages and probably got into plants as a result of horizontal transfer [37, 38]. These polymerases recognize different promoters: PEP promoters resemble bacterial ones, and NEP promoters bear a resemblance to promoters in the plant mitochondrial genome. On the whole, PEP promoters are much more powerful than NEP promoters and the majority of intensely transcribed genes in plastids are controlled by PEP promoters [39]. Therefore, PEP promoters are preferable for biosynthesis of transgenes. The strongest promoter in plastids of higher plants (Prm) controls the operon of ribosomal RNAs and specifically Prm was used for production of numerous most intensely expressing cassettes. Since rRNA is not translated, Prm must be supplemented with an appropriate signal of the beginning of translation for intense expression of transgene [40]. Operon \( \text{psbA} \) is also very promising for transgenosis; it encodes protein D1 of photosystem II, which is not only intensely transcribed but its transcripts are actively translated, which sometimes results in higher levels of expression than in the case of \( \text{Prm} \) [41]. In addition to plastid promoters, powerful bacterial or mitochondrial promoters are sometimes used for control over expression of transgenes in plastids. Although they are recognized by plastid transcription machinery, they are much weaker than plastid PEP promoters [42, 43].

**Regulatory elements: 5’- and 3’-UTRs.** Since gene expression in plastids is for the most part regulated at the level of translation [44, 45], it is not surprising that the correct choice of 5’- and 3’-UTRs should be very important for active expression of the transgene in plastids. By the level of expression of reporter genes, not only numerous plastid but also some exogenous 5’-UTRs were tested (for the most part, bacterial and even synthetic) [46]. One of the strongest factors initiating translation turned out to be 5’-UTR of \( \text{gene10} \) from bacteriophage T7 [47]. In combination with plastid promoter \( \text{Prm} \), it ensures one of the highest levels of accumulation of transgenic proteins [47, 48]. A very high level of expression of transgenes is ensured by promoter/5’-UTR of plastid gene \( \text{psbA} \) encoding the most intensely translated plastid protein [49]. Other endogenous regulatory sequences (for instance, of genes \( \text{rrn} \), \( \text{psbA} \) are used, for instance, \( \text{psbA} \) from lettuce in tobacco chloroplasts and vice versa. Differences in the sequences of 5’-UTR of tobacco and lettuce result in a pronounced decrease in the affinity of 5’-UTR to mRNA-binding proteins [41]. This once again demonstrates that, in order to ensure high levels of transgene expression in plastids, species-specific vectors should be engineered.

3’-UTRs of plastidal mRNA produce stable loops in the secondary structure, which protect mRNA from quick degradation and ensure its accumulation [50]. Numerous investigations of the role of plastid 3’-UTRs indicate that the choice of 3’-UTR affects the level of mRNA accumulation, but their contribution to transgene expression is not notable [51, 52]. This agrees with the fact that the regulation of gene expression in
plastids predominantly occurs at the translational level and the mechanisms regulating translation may cope with even high levels of mRNA [52, 53].

Thus, the level of expression of target gene in transplastomic plants depends a lot on numerous factors, out of which the main are correct choice of the insertion site, promoter, and regulatory untranslated regions 5'- and 3'-UTRs. As an example, Table 2 shows variability of the accumulation of recombinant proteins, with the first six lines corresponding to the optimal combination of regulatory elements and integration site (trnI/trnA).

The shown data suggest that the accumulation of recombinant protein varies from 0.85 to 45.0% of total soluble protein (TSP) even in the case of insertion into the same site. Actually, this range may be even wider. The level of expression may also depend substantially on quite different factors, for instance, on the accuracy of selection of homoplastomic and homoplastidic forms of transgenic plants.

| Insertion sites | Promoter/5'-UTR/Terminator(3'-UTR) | Efficiency of expression, % of TSP |
|----------------|-----------------------------------|----------------------------------|
| trnI/trnA      | PpsbA/TpsbA                       | 32–38; 17–26                    |
| trnI/trnA      | Prrn/TpsbA                        | 45.3                             |
| trnI/trnA      | TrbcL                             | 10.0                             |
| trnI/trnA      | Prrn/TpsbA                        | 0.85–1.0                         |
| trnI/trnA      | PpsbA/TpsbA                       | 5.16–9.27                        |
| trnI/trnA      | PpsbA/TpsbA                       | 0.2–6.0                          |
| trnV/rps12,7   | Prrn/TrbcL                        | >7.0                             |
| rbcL/accD      | PpsbA/rbcL.3’                     | 5.0                              |
| rps7,12/trnV   | Prrn/T’g10/Trps16                 | >10.0                            |
| rbcL/accD      | Prrn/TpsbA                        | 2.0–3.0                          |
| trnfM/trnG     | Prrn/T’g10/rbc3’                  | 0.8–1.6                          |

Table 2. Expression of some transgenes depending on the insertion site, used promoters, and regulatory regions (quoted from [30], with modifications)

PRODUCTION OF HOMOPLASTOMIC AND HOMOPLASTIDIC PLANTS

One of the main problems associated with the production of transplastomic plants with a high level of transgene expression is difficulty in obtaining cells where all the plastomes of all the plastids bear the transgene and out of which homoplastomic and homoplastidic plants may be subsequently regenerated. During two recent decades, the methods of production of homoplastomic and homoplastidic plants have not changed much and are still based on a multistep screening with the use of two or more selectable factors. The general pattern of plastid genome transformation as a result of multistep screening on selective media is shown in Fig. 1. An ideal result ensuring the highest levels of expression of target genes is the production of homoplastomic and homoplastidic cells (Fig. 1e).

Recently, this selective pattern was supplemented with a tendency to transform not mature chloroplasts but proplastids of meristematic and etiolated callus tissues, which primarily contain a small number of plastids with few plastomes.

Use of meristematic and etiolated callus tissues. Chloroplast plastomes are organized as nucleoids: rather compact nucleoprotein complexes located on thylakoid membranes of chloroplasts. The number of plastomes in each nucleoid may vary from one to 20 and more depending on the type of tissue. Apical meristematic tissues contain a small number of proplastids and immature chloroplasts bearing approximately ten plastomes, whereas mature chloroplasts of leaf mesophyll contain 70–130 copies of plastome, up to 2600–3300 copies per cell [54, 55]. In etiolated cell and callus cultures, chloroplasts dedifferentiate and turn into proplastids; the number of plastomes therein sharply decreases—the same as the number of plastids per cell [55–57]. Upon transformation of chloroplasts, foreign DNA with target and selective genes is incorporated only in a small number of cell plastomes. Use for transformation of meristematic and etiolated callus cultures considerably reduces the number of plastomes that remained intact, which subsequently greatly facilitates selection for homoplastomy and homoplastidity. Transformation of callus cells resulted in the production of transplastomic plants of *A. thaliana* and sugarcane, although homoplasmity of these plants is not evident in some cases [58–60].

Screening and selectable markers. Since the majority of cell plastomes remain intact as a result of transformation, the attainment of homoplastomy and homoplastidity always requires a multistep screening by one or two selectable markers. The production of transplastomic plants depends on different genes that
ensure resistance to various antibiotics. The majority of tested antibiotics are selective inhibitors of protein synthesis on the ribosomes of a prokaryotic type (70S) and do not affect protein synthesis on 80S ribosomes in the cytoplasm. These antibiotics include spectinomycin, streptomycin, kanamycin, and chloramphenicol. They are often used for the initial steps of screening when only a small number of plastomes bear a selectable marker [61]. Expression of the genes of neomycin phosphotransferase (nptII) or aminoglycoside 3'-phosphotransferase (aphA6) ensures resistance to kanamycin [62, 63]. The most often used in plastid transformation selectable marker is the gene of streptomycin 3'-adenylyltransferase (aadA), which ensures plastid resistance to two antibiotics: streptomycin and spectinomycin [64, 65]. Spectinomycin binds with plastid ribosomes and blocks protein translation [66]. Screening on the media containing these two antibiotics (streptomycin + spectinomycin) is performed for the separation of transplastomic clones from rather often arising spontaneous mutants resistant to spectinomycin [61]. Tolerance to chloramphenicol ensured by the gene of bacterial chloramphenicol acetyltransferase (CAT) is less pronounced than resistance to kanamycin or spectinomycin but its advantage consists in the absence of tolerant spontaneous mutants [67]. Selection of transplastomic clones by their resistance to 4-methylindole and 7-methyl-DL-triptophan (gene of anthranilate synthase, ASA2) [68] and to betaine aldehyde (gene of betaine aldehyde dehydrogenase, badh) was also efficient [69].

In early stages of screening when the number of transformed plastomes is still small, it is necessary to take into account such a common phenomenon in plastids as gene conversion. If the target gene is loosely linked to the selectable marker, it may be lost with preservation of the resistance to the selective agent as a result of gene conversion since the majority of template plastomes do not bear the target gene [70]. Therefore, it is very important to promptly introduce transplastomic plants into additional cycles of selec-

![Fig. 1. Reorganization of plastid genome as a result of screening on selective media for attaining homoplastomy and homoplasidity. (a) Introduction of a genetically engineered construct (transgene) into plastids (chloroplasts) by any available method, for instance, by means of biolistics; (b) incorporation of one or several copies of the transgene into the native genome (plastome) of chloroplasts by means of homologous recombination; (c–f) screening of cells on the medium with a selective agent (as a rule, antibiotic spectinomycin or a mixture of spectinomycin and streptomycin). Designations: green ovals within the cell are native plastids with untransformed genome; bicolor ovals within the cell are plastids containing transgenic and conventional copies of genome (heteroplastidic chloroplasts); red ovals within the cells are plastids containing only transformed copies of genome (homoplastidic chloroplasts); bubbles above the cells show individual nucleoids of plastids containing only conventional copies of the genome (green circles); nucleoids containing a mixture of transgenic and conventional copies of the genome (combination of green and red circles); and nucleoids containing only transgenic copies of genome (red circles); arrows with caption “screening” designate culturing of tissues, calli, or cells on the media supplemented with selective agents.](image-url)
tion in order to ensure their stable homoplastomic state and analyze a large number (dozens) of regenerants in each regeneration cycle by means of dot blot technique or the PCR method in order not to lose the target gene [9].

In the late stages of selection when the number of transformed copies of plastome becomes large, it is advisable to use such secondary selectable markers as bar that ensures resistance to herbicides phosphinothricin and glyphosate [71] and the genes of resistance to sulfonylurea, pyrimidinylcarboxylate [72], and diketonitrile [73].

Not long ago, a new and peculiar method of plastid transformation was proposed that ensured efficient homoplastomy [74]. The method is based on the use of a barnase-barstar pair of genes from Bacillus amyloliquefaciens [75]. The barnase gene encodes ribonuclease that produces a cytotoxic effect, and the product of the barstar gene specifically inhibits it [76]. The authors fused barnase gene with a signal of transport to plastids and put it under control of a β-estradiol-based inducible system [77]. This construct was incorporated into the nuclear genome of tobacco, and plastids were transformed by a vector bearing the barstar gene and reporter gene GFP. Upon induction with estradiol, only plastids transformed with barstar survived, which allowed the researchers to quickly obtain homoplastomic and homoplastidic tobacco plants [74].

CONCLUSIONS

Transformation of plastids undoubtedly opens up new horizons in plant biotechnology and bioengineering. As compared with nuclear transformants, transplastomic plants have a lot of advantages. They consist in a potentially high yield of desired protein, an opportunity of precise incorporation of the target gene into a predetermined region of the plastome via the mechanism of homologous recombination, and the possibility of transfer of gene clusters responsible for entire metabolic pathways by means of producing artificial operons. Accommodation of target genes in plastids essentially rules out their silencing and minimizes the position effect. Moreover, transplastomic plants are more reliable in terms of biological security since plastids are not transferred with pollen to other plants. Thus, transplastomic plants may promote a considerable break-through in biotechnology within the range from creation of drugs to a large-scale commercial synthesis of enzymes and biofuel. Meanwhile, serious problems arise on this path; for the most part, they reduce to low frequency of plastid transformation and difficulties with the selection of homoplastomic and homoplastidic plants. Pathways to the solution of these problems are evident. The first problem may be solved by means of developing new systems of delivery of foreign genes to plastids and a fine design of transforming vectors. The system of gene delivery via nanotubes makes it possible to elevate the frequency of delivery to plastids and probable integration into the plastome more than tenfold. Since integration obeys the HR mechanism, it is crucial that flanking sequences should have total homology to the sequences of the insertion site. Thus, it is necessary to engineer a special construct for each plant species. This also applies to promoter and regulatory 3’- and 5’-UTRs that should be species-specific, which improves the yield of the desired protein. Difficulties with attaining homoplastomy may be overcome via creation of novel systems of selection (for instance, barnase/barstar system) and use for transformation of meristematic and dedifferentiated cells with a small number of plastomes.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving humans or animals performed by any of the authors.

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