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EXPERIMENTAL ARTICLES

The Transformation of the Unicellular Alga *Chlamydomonas reinhardtii* by Electroporation

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Abstract—The cell wall–lacking mutant CW-15 of the unicellular green alga *Chlamydomonas reinhardtii* was transformed by electroporation using plasmid pCTVHyg, which was constructed with the hygromycin phosphotransferase gene *hpt* as the selective marker and the Tn_5 transposon of *Escherichia coli* under the control of the virus SV40 early gene promoter. Under optimal conditions (10^6 mid-exponential cells/ml; electric field strength 1 kV/cm; and pulse length 2 ms), the transformation yielded 10^3 Hyg^R transformants per 10^6 recipient cells. The exogenous DNA integrated into the nuclear genome of *Ch. reinhardtii* was persistently inherited through more than 350 cell generations. The advantages of this system for the transformation of *Ch. reinhardtii* with heterologous genes are discussed.

Key words: plasmid pCTVHyg, hygromycin phosphotransferase, transformation, electroporation, mutant CW-15, *Chlamydomonas reinhardtii*.

Chlamydomonas reinhardtii is a convenient object to study biological processes such as the functioning of chloroplasts, cell interactions, mating, the life cycle of cells, respiration, and photosynthesis. Due to its ability to grow autotrophically in low-cost mineral media, this alga could be a potential producer of valuable foreign proteins, provided that the problem of the unstable expression of heterologous genes in this alga is solved.

The nuclear genome of *Ch. reinhardtii* is typically transformed by foreign genes using the bacterial kanamycin phosphotransferase gene *nptII* as the selectable marker [1]. This is not a good choice, since *Ch. reinhardtii* is characterized by a high rate of spontaneous mutations with respect to kanamycin resistance [1, 2] and a low yield of transformants [3]. For this reason, I attempted to use the hygromycin phosphotransferase gene *hpt* as the selective marker (to solve the problem of kanamycin resistance mutations) and the cell wall–lacking mutant CW-15 (to fix the problem of low transformation rates upon electroporation).

It should be noted that considerable progress in the transformation of the *Ch. reinhardtii* chloroplasts was largely due to Boynton *et al.* [4], who employed bombardment with tungsten microparticles covered with plasmid DNA (the so-called biolistic technique), whereas the nuclear genome of *Ch. reinhardtii* was transformed at a high frequency through the intense grinding of a cell suspension in the presence of glass beads and plasmid DNA (the so-called glass-beads technique) [5]. In these and other works, the authors used the chloroplast and nuclear homologous genes of *Ch. reinhardtii* for transformation.

The stable transformants of *Ch. reinhardtii* were also obtained through the restoration of the wild-type sequences of the mutant chloroplast genes *atpB* [4], *rbcL* and *psbA* [6], and *psaB* [7]. This approach is based on homologous recombination between a mutant gene and a wild-type cloned sequence. The mutant chloroplast genes of 16S rRNA and 23S rRNA, which determine resistance to spectinomycin, streptomycin, and erythromycin, were used for the selection of transformants resistant to these antibiotics [6]. The nuclear DNA of *Ch. reinhardtii* was successfully transformed by the homologous genes *nit1* [5], *oee1* [1], *rsp3* [5], and *arg7* [8].

There is little information in the literature on the transformation of the nuclear genome of *Ch. reinhardtii* with heterologous genes. Thus, researchers reported on the stable expression of the *Escherichia coli* β-glucuronidase gene *uidA* fused with the 5'-nontranslatable region of the chloroplast gene *petD* of *Ch. reinhardtii* [9] and the *E. coli* aminoglycoside adenyltransferase gene *aadA* encoding resistance to spectinomycin and streptomycin in *Ch. reinhardtii* [10]. Nevertheless, the problem of the stable expression of heterologous genes in the nuclear genome of this green alga is to be completely solved, since little is known about the causes of the unstable expression of such genes.

The genome of *Ch. reinhardtii* was first transformed using the yeast arginine succinate-lyase gene *arg4* to complement the mutant ARG7 of this alga [3] and the neomycin phosphotransferase gene *nptII* of the bacte-

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rial Tn5 transposon as the selective marker [1]. The use of these selective systems posed some problems, which were discussed by Mayfield and Kindle [1] and Hall *et al.* [2]. The *nptII* gene was expressed in 52% of clones transformed with a plasmid bearing both *nptII* and the second selective marker *nit1* (selection was carried out in a medium with nitrate as the sole nitrogen source) [2]. Some problems in the transformation of *Ch. reinhardtii* with foreign genes are related to the use of kanamycin resistance as the selective marker [2], since this alga exhibits a high frequency of kanamycin resistance mutations.

The aim of this work was to construct plasmid pCTVHyg and to transform the cell wall–lacking mutant CW-15 of *Ch. reinhardtii* by means of electroporation with the use of the *E. coli* hygromycin phosphotransferase gene *hpt* as the selective marker.

MATERIALS AND METHODS

Algal strain, medium, and cultivation conditions. Experiments were carried out with the cell wall–lacking mutant CW-15 of the wild-type strain 137 C mt^+ of *Ch. reinhardtii* Dang [11]. The mutant was cultivated either in a liquid mineral medium described earlier [12] or on the same medium solidified with 1.5% agar (Difco, United States). Cultivation in the liquid medium was carried out at 25°C under 16000-lx illumination and intense aeration with the air containing 5% CO_2.

Transformation procedure. Cells of the mutant CW-15 were transformed by electroporation using a device generating rectangular electric pulses (Medical Academy, Kaunas, Lithuania). The mutant was grown to a density of 10^6 cells/ml. Cells were harvested by centrifugation at 1500 *g* for 5 min, washed in the fresh cultivation medium, and resuspended to a density of 10^8 cells/ml in the electroporation buffer (10 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.4 M mannitol). Cells were counted microscopically using a Goryaev chamber. The cell suspension was supplemented with 5 μg plasmid DNA and 10 μg salmon sperm DNA (the control suspension did not contain plasmid DNA). Aliquots (0.4 ml) of the control and experimental cell suspensions were placed in an electroporation chamber with electrodes 1 cm apart and subjected to the action of an electric pulse (field strength 1 kV/cm; pulse length 2 ms). Then cells were kept on ice for 10 min, incubated in light for 16–18 h, precipitated by centrifugation at 1500 *g* for 5 min, and resuspended in 1 ml of the cultivation medium. Aliquots (0.1 and 0.5 ml) of the cell suspensions were mixed with soft agar (minimal medium with 0.6% Difco agar and 10 μg/ml hygromycin B) and poured onto the solid 1.5% agar medium supplemented with 10 μg/ml hygromycin B (the antibiotic was purchased from Calbiochem, United States). The plates were incubated at 25°C in light for 10–14 days.

The isolation of the total DNA and Southern hybridization. The total DNA was isolated from 20 ml of a culture grown to the late exponential growth phase. Southern hybridization was carried out as described in [13] using a nylon filter (Hiiu Kalur, Estonia), the *Bam*HI fragment of plasmid pPCV730 containing the coding region of the reporter *hpt* gene, and random primers purchased from Boehringer Mannheim (Germany). Plasmid pPCV730 was kindly provided by J. Shell, Max Planck Institut für Zuchtungsforschung, Köln, Germany.

Assay of neomycin phosphotransferase II. Transformed CW-15 cells were suspended in 10 ml of the liquid mineral medium to a density of 10^6 cells/ml, incubated in light for 18 h, and analyzed for the activity of neomycin phosphotransferase II (NPT-II) as described by Reiss *et al.* [14].

RESULTS

Construction of plasmids. Two integrative vectors (Fig. 1) were constructed using heterologous genes and the hygromycin phosphotransferase gene *hpt* [15] and the neomycin phosphotransferase gene *nptII* from the *E. coli* Tn5 transposon [1, 16] as the selective markers.

The transformation vector pCTVHyg (CTV stands for "*Chlamydomonas*-transforming vector") was constructed with the hygromycin (Hyg) phosphotransferase gene *hpt* as the selective marker (Fig. 1). The starting plasmid pSV2Cat [11] was digested with *Hin*dIII and *Hpa*I restriction enzymes. A 3.5-kb restriction fragment containing the virus SV40 polyadenylation site and the promoter was ligated with a 1.1-kb *Bam*H1 restriction fragment of plasmid pPCV730 with the coding region of the *hpt* gene [15]. Sticky ends were preliminarily filled in with the Klenow fragment of DNA polymerase I. *E. coli* strain HB101 was transformed with the ligase mixture, and bacterial clones with the direct orientation of the *hpt* gene were selected. These clones contained plasmid pCTVHyg. All the manipulations were carried out as described by Maniatis *et al.* [17].

The transformation vector pCTVNeo (Fig. 1) was constructed with the neomycin (Neo) phosphotransferase gene *nptII* as the selective marker of kanamycin resistance. The starting plasmid pSV232A-L [11] was digested with *Hin*dIII and *Sma*I restriction enzymes. A 6.6-kb restriction fragment containing the virus SV40 early gene promoter and the polyadenylation site was ligated with a 1.0-kb *Bgl*II–*Bam*HI restriction fragment of plasmid pMON129 containing the coding region of the *nptII* gene [16]. Sticky ends were preliminarily filled in with the Klenow fragment of DNA polymerase I. The *E. coli* strain HB101 was transformed with the ligase mixture, and bacterial clones with the direct orientation of the *nptII* gene were selected. These clones contained plasmid pCTVNeo.

The transformation of the *Ch. reinhardtii* mutant CW-15 cells. These cells were transformed by electroporation [11] using plasmid pCTVHyg, which contained the coding region of the *hpt* gene under the control of the virus SV40 early gene promoter. The transformation was most efficient at an electric field strength of 1 kV/cm and a pulse length of 2 ms (Fig. 2).

After 4–5 days of incubation in the light on the selective agar, hygromycin-sensitive (Hyg^S) cells died out (this could easily be seen under a microscope), whereas hygromycin-resistant (Hyg^R) cells gave rise to colonies, which became visible after 8–10 days of incubation. The efficiency of transformation corresponded to 10^3 Hyg^R clones per 10^6 recipient cells and was maximum with midexponential cells (16–30 h of growth; culture density 10^6 cells/ml) (Fig. 3). The transformation efficiency of mutant cells taken from cultures containing less than 0.5×10^6 or more than 2.5×10^6 cells/ml dramatically fell (Fig. 3).

Analysis of transformants. After 10 months of regular culture transfers to fresh nonselective growth media at 2-week intervals, four Hyg^R clones were subjected to analysis of their DNA. To this end, the total DNAs of these clones were digested with *Pst*I restriction endonuclease and the digestion products were separated in 0.8% agarose gel. Then the products were transferred onto a nylon filter and hybridized with a *Bam*HI restriction fragment of plasmid pPCV730 containing the coding sequence of the *hpt* gene (Fig. 1). In the case of two clones, *Pst*I digested plasmid pCTVHyg into three fragments, 2.7, 1.0, and 0.9 kb in size, two of which (2.7- and 1.0-kb) were homologous to the probe.

In spite of the fact that plasmid pCTVHyg integrated into the nuclear genome of *Ch. reinhardtii* was persistently inherited through more than 350 generations, the phenotypic character of hygromycin resistance was very unstable, as is evident from the analysis of the population of two Hyg^R clones (H-1 and H-2). Indeed, cells of these clones taken from cultures with densities of 10^6 cells/ml and plated onto the hygromycin-containing selective medium with a serial dilution factor of 1.3 beginning from the inoculation dose of 10^4 cells per 120-mm petri dish produced Hyg^R colonies, whose relative number comprised only 0.1–1% of the total number of plated cells (data not presented). In addition to distinct Hyg^R colonies of normal size, the plates also contained a large number of small colonies with nonviable cells and tiny colonies with dead cells (the latter were visible only under a microscope). The results were almost the same with freshly prepared transformants and with transformants subcultured for 8–10 months. It should be noted that cells of the untransformed mutant CW-15 plated onto the hygromycin-containing selective medium completely died after 4–5 days of incubation.

As is evident from Fig. 4, the *hpt* gene was present in the *Ch. reinhardtii* genome in more than one copy. As is known from the genetics of higher plants, the integration of a heterologous gene in a nuclear genome, as a rule, leads to its silencing. This phenomenon could also be responsible for the instability of the *hpt* gene in the *Ch. reinhardtii* genome. Another cause of instability of Hyg^R transformants could be due to the *hpt* gene rearrangement, which is evident from the fact that the DNA of the transformants contains some nucleotide sequences typical of the *hpt* gene but not present in plasmid pCTVHyg (Fig. 4).

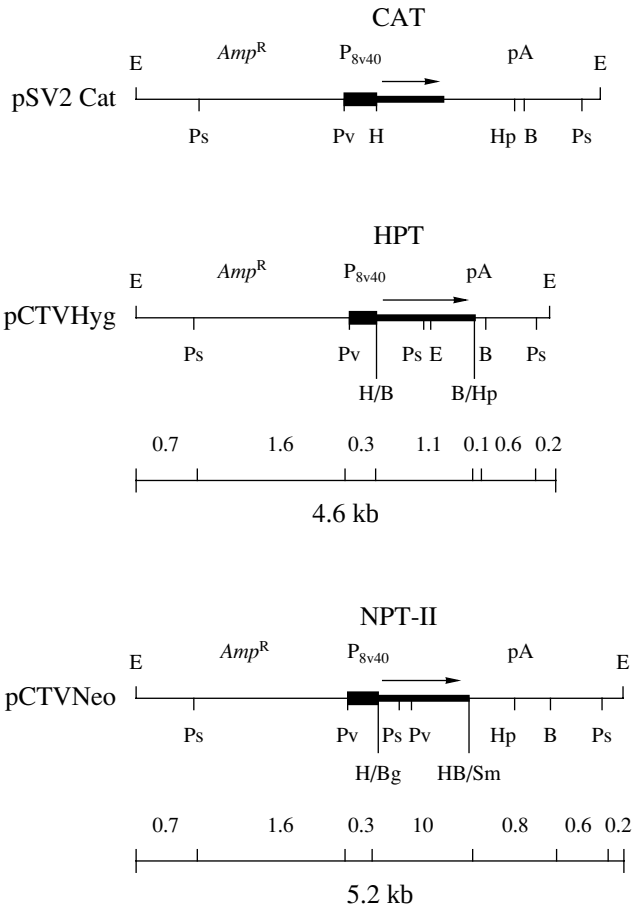

Fig. 1. The structure of the starting plasmid pSV2Cat and its derivatives pCTVHyg and pCTVNeo linearized with respect to the *Eco*RI site. CAT, chloramphenicol acetyltransferase; HPT, hygromycin phosphotransferase; NPT-II, neomycin phosphotransferase; *Amp*R, ampicillin resistance; P_{SV40}, the early gene promoter of virus SV40; pA, the polyadenylation site of virus SV40; E, *Eco*RI; Ps, *Pst*I; Pv, *Pvu*II; H, *Hin*dIII; Hp, *Hpa*I; B, *Bam*III.

Comparison of the codon usage frequencies of the *Ch. reinhardtii* nuclear genes and the *hpt* gene. The nuclear genes of *Ch. reinhardtii* are characterized by the so-called biased codon usage [1, 2]; namely, nucleotide triplets with A at the third place are used with a very low frequency. This phenomenon may hinder the expression and cause the instability of foreign genes in *Ch. reinhardtii* cells [1]. Hall *et al.* [2] calculated the codon usage frequency of the 15 nuclear genes of *Ch. reinhardtii* and compared them with that

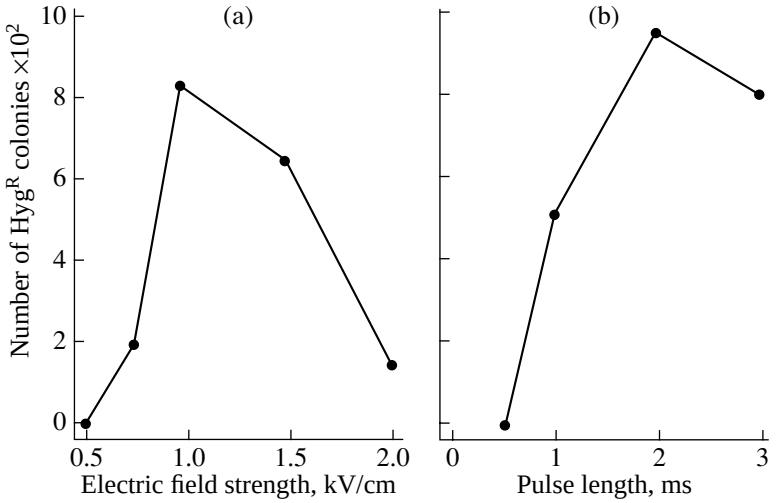

Fig. 2. The dependence of the transformation efficiency on (a) the electric field strength at the pulse length equal to 2 ms and (b) the pulse length at the electric field strength equal to 1 kV/cm.

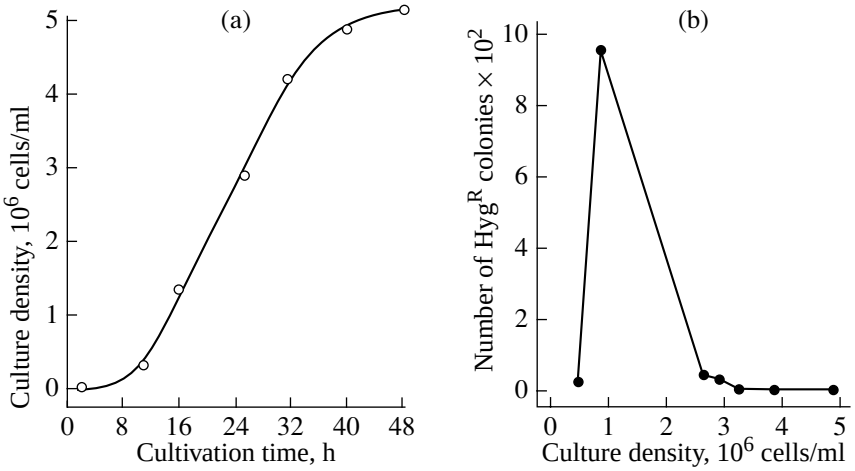

Fig. 3. (a) The growth curve of mutant CW-15 in a mineral medium and (b) the dependence of the transformation efficiency in the presence of plasmid pCTVHyg on the growth phase of mutant cells (specifically, on the density of the culture from which these cells were obtained).

of the *hpt* gene (A. Franco "*Chlamydomonas* Newsletters," August 1993). In this work, I compared the codon usage frequencies of the *hpt* gene [15] and those of five nuclear genes of *Ch. reinhardtii* with the aid the computer program Microgenie and found that some triplet codons with A at the end (TTA, CTA(Leu), and TCA(Ser)) are not used, while the other such codons are used with frequencies varied from 0.3 to 3.2%. Consequently, in the experiments described in this paper, biased codon usage is unlikely to be responsible for the instability of the *hpt* gene in the *Ch. reinhardtii* genome.

Analysis of the transient expression of the *nptII* reporter gene. The mutant CW-15 cells were transformed by electroporation under optimal conditions (1 kV/cm; 2 ms) with plasmid pMON129 containing the recombinant neomycin phosphotransferase *nptII* gene, whose coding region was fused with the regulatory sequences of the nopaline synthase [11]. After incubation for 18 h, the crude extract of CW-15 cells was analyzed for the activity of NPT-II (Fig. 5). This activity was found to be present in all variants with pMON129 (lanes *2*, *3*, *4*) and to be absent in the control variant (electroporation without plasmid DNA, lane *5*). Consequently, the *nptII* gene can be used as a reporter gene for the study of transient expression from heterologous promoters in *Ch. reinhardtii* cells. The use of this gene can likely help to solve the problem of the unstable expression of heterologous genes when they are integrated into the nuclear genome of algae and are inherited through many cell generations.

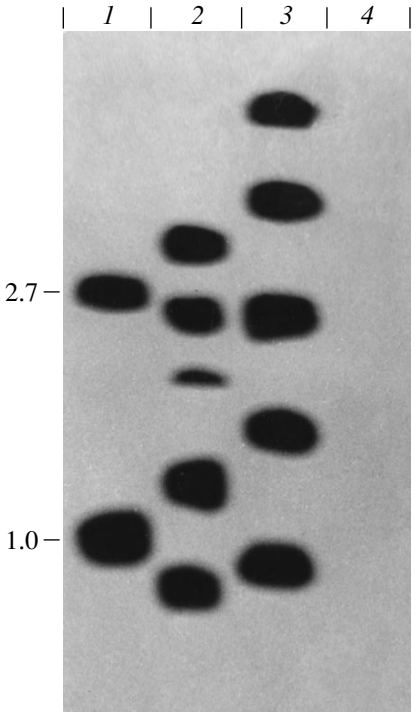

Fig. 4. The hybridization of the total DNA of (*1*) plasmid pCTVHyg, (*2, 3*) the transformed clones H-1 and H-2, respectively, and (*4*) the untransformed mutant CW-15. The total DNA was digested with *Pst*I restriction endonuclease. The digestion products were separated in 0.8% agarose gel, transferred onto a nylon filter, and hybridized with a *Bam*HI fragment of plasmid pPCV730 containing the coding region of the reporter gene *hpt*. The amount of the DNA of CW-15, H-1, and H-2 taken for hybridization was 5 μg and that of plasmid pCTVHyg was 1 μg.

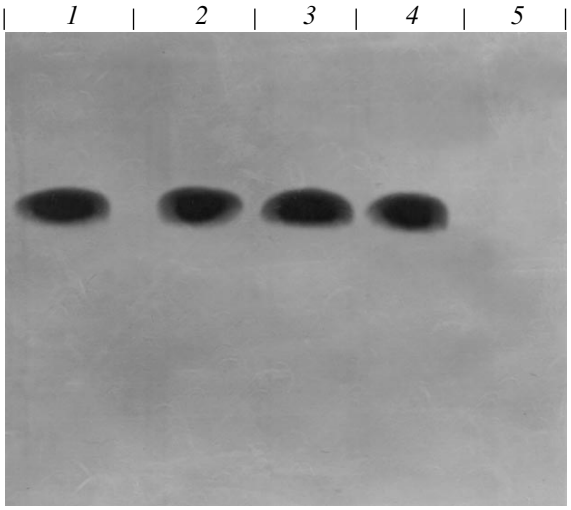

Fig. 5. Analysis of the transient expression of NPT-II. Lanes: (*1*) extract of *E. coli* cells producing NPT-II (positive control); (*2, 3, 4*) extract of *Ch. reinhardtii* cells transformed with plasmid pMON129; and (*5*) extract of the untransformed CW-15 mutant cells (negative control).

DISCUSSION

The expression of heterologous genes is one of the central problems of the genetic transformation of *Ch. reinhardtii*. The *hpt* gene as a new selective marker of hygromycin resistance has some advantages over the *nptII* gene of kanamycin resistance. First, cells of the mutant CW-15 are extremely sensitive to low concentrations of hygromycin and die out in soft agar containing as little as 10 μg/ml hygromycin after 4–5 days of incubation. Second, the spontaneous mutations of hygromycin resistance are very rare. For instance, none of the 10^8 *Ch. reinhardtii* cells plated on the hygromycin-containing selective medium in this work gave rise to Hyg^R colonies.

The transformation of the CW-15 mutant cells by the optimized method of electroporation [11, 18] makes it possible to obtain 10^3 Hyg^R transformants per 10^6 recipient cells. Such transformation efficiency of *Ch. reinhardtii* is close to that showed by the glass-beads approach [5] and two orders of magnitude higher than the efficiency of transformation by electroporation reached earlier [3, 19].

The maximum yield of transformants in this work was observed with mid-exponential cells, which can be explained by the intense synthesis of DNA in such cells. This finding disagrees with the results of Kindle [5], who obtained transformants of even stationary-phase *Ch. reinhardtii* cells, in which the activity of DNA synthesis drastically declines.

The analysis of Hyg^R clones showed that the foreign DNA integrated into the nuclear genome of *Ch. reinhardtii* is persistently inherited through a large number of generations; i.e., it is mitotically stable. The possibility of the autonomous replication of plasmid pCTVHyd should be excluded, since it is known that the bacterial sequences of replication initiation are unable to maintain the plasmid in the *Ch. reinhardtii* cytoplasm and that plasmid DNA introduced into a cell by means of electroporation rapidly degrades [19]. The only known case of the autonomous replication of plasmid DNA containing the *ori* gene of the yeast plasmid 2μm (*ori*-2μ) was not confirmed in other laboratories. Even the plasmids that contain the autonomously replicating sequences (the so-called ARS elements) of *Ch. reinhardtii* chloroplasts are unstable and completely degrade after 80–100 cell divisions under selective conditions [20]. Therefore, the results presented here confirm the earlier observations [2, 3, 9, 10] that the heterologous sequences of *Ch. reinhardtii* are stable. Nevertheless, the phenotypic character of hygromycin resistance is very unstable, as is evident from the analysis of the population of Hyg^R clones subcultured on the selective medium for 10 months.

Generally speaking, the instability of the hygromycin resistance character can arise at three different levels (the levels of DNA, RNA, and protein) and can be accounted for by (1) the instability of the integrated

sequence of a foreign gene, (2) the effect of some factors on the transcription and processing of mRNA, and (3) the effect of some factors on the synthesis and stability of the protein product of the foreign gene. It should also be taken into account that the nucleus and the chloroplast (if pCTVHyg plasmids are integrated into both the nuclear and chloroplast genomes) can differ in stability of the phenotypic manifestation of hygromycin resistance.

There is information in the literature on the structural stability of foreign genes, their transcription, and the activity of the protein products of these genes in *Ch. reinhardtii* cells. For instance, it is known that the *uidA*, *aadA*, and *nptII* gene sequences integrated into the chloroplast genome and the *nptII* gene sequence integrated into the nuclear genome can be steadily inherited in the process of mitosis [2, 9, 10]. One of the possible causes of the phenotypic instability of the hygromycin resistance character (namely, biased codon usage) is likely to be excluded. This follows from the analysis of the codon usage frequencies for the *nptII* gene [2] and the *hpt* gene (the data of this work). According to recent observations, the foreign β-glucuronidase and aminoglycoside adenyltransferase encoded by the *uidA* and *aadA* genes, respectively, retain their activity in *Ch. reinhardtii* chloroplasts [9, 10]. Similarly, the foreign neomycin phosphotransferase encoded by the *nptII* gene remains active in the *Ch. reinhardtii* cytoplasm [2].

There is evidence that the stability of heterologous proteins in some organisms is genetically determined. In particular, *E. coli* has the protease gene *lon*, whose inactivation augments the stability of foreign genes in this bacterium. Some mutations in the yeast *Saccharomyces cerevisiae* act in the same manner [11]. Provided that such mutations are possible in *Ch. reinhardtii* as well, the stability of the hygromycin resistance character in this alga can likely be enhanced by selecting clones with an elevated level of hygromycin resistance and a more stable phenotypic manifestation of this character. It should be noted in this regard that the selective system used in this work has two important advantages: a high yield of Hyg^R transformants and the absence of spontaneous mutation with respect to hygromycin resistance.

Thus, experimental data indicate that the heterologous DNA integrated into the nuclear and chloroplast genomes of *Ch. reinhardtii* is stable and may be expressed with the synthesis of active foreign proteins. The observed instability of Hyg^R transformants may be due to the rearrangement of integrated plasmid DNA in the course of the long-term subculturing of the transformants and the simultaneous integration of several copies of the *hpt* gene, which gradually leads to gene silencing. The possibility that the instability of foreign proteins lies in their susceptibility to the proteases of host cells also cannot be excluded. Extensive genetic studies may lead to a breakthrough in this line of research.

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