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Investigation of an Optimized Context for the Expression of GFP as a Reporter Gene in *Chlamydomonas Reinhardtii*

M. Ghanbari Motlagh, Z. Amini-Bayat* and H. Ofoghi*

Department of Biotechnology, Iranian Research Organization for Science and Technology, Tehran-Iran

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ABSTRACT

Chlamydomonas reinhardtii is a novel recombinant eukaryotic expression system with many advantages including fast growth rate, rapid scalability, absence of human pathogens and the ability to fold and assemble complex proteins accurately, however, obstacle relatively low expression level necessitates optimizing foreign gene expression in this system. The Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* is a substantial reporter molecule for monitoring gene expression and protein localization.The fluorescence of GFP requires only UV or blue light, and, therefore, the *in vivo* observation of GFP expression is easy with no need for complex and costly apparatus.

Keywords: *Chlamydomonas reinhardtii*, Green Fluorescent Protein, Optimization, Recombinant protein expression

INTRODUCTION

The single-celled eukaryotic microalgae *Chlamydomonas reinhardtii* has emerged as a powerful platform for studying cellular processes including the flagellar structure and function, lipid metabolism and photosynthesis [1-3]. It is also important for producing therapeutic proteins [4]. *C. reinhardtii* has the ability to grow heterotrophically, photoautotrophically or mixotrophically in addition to sexual or asexual reproduction [5,6]. Recently, researchers have shown an increased interest in using *C. reinhardtii* due to its numerous advantages such as well identified genetics, rapid growth, being eukaryotic, short time from transformation to product, ability in post translational modification like mammalian cells, rapid scalability at bioreactors and a low production cost. All three genomes (nucleus, chloroplast and mitochondria) of *C. reinhardtii* have been completely sequenced, and to date various methods have been developed and introduced for the transformation of *C. reinhardtii* [7-10]. One of the main obstacles of *C. reinhardtii* as a recombinant expression system is its poor expression of foreign genes especially in the nucleus. Little is known about this obstacle in *C. reinhardtii*, however, some possible reasons for a poor nuclear expression such as gene silencing by yet unknown epigenetic effects, inappropriate codon usage, lack of suitable regulatory sequences for transcription and translation are considered [6,11]. A number of approaches such as using appropriate promoters [12-15], codon optimized genes [6,16,17], insertion of intronic sequences [18,19] in transgenes have proven useful.

Nowadays reporter genes have been used widely in different fields like biotechnology, molecular biology and biomedicine [20]. Green fluorescent protein (GFP) is a well characterized reporter gene which can be easily detected and quantified. GFP is derived from the jelly fish *Aequorea victoria*. Natural GFP has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm [21,22]. GFP, as a reporter gene, has been expressed in the nuclear and chloroplast genome of *C. reinhardtii* [11,23]. Unmodified GFP gene under the control of heterologous promoters demonstrated poor expression [11]. The presence of A/T rich codons in the native gene is a reason for poor expression. To overcome this restriction, cGFP gene was synthesized. cGFP was modified based on codon preference of *C. reinhardtii*. The cGFP is a nuclear codon-optimized gene that is used for

*Corresponding author. E-mail: Amini-bayat@irost.ir

expression from the nuclear genome of *C. reinhardtii*. In this research we attempt to show the effect of codon optimized gene and other approaches such as using hybrid promoter, intron and appropriate 3′ UTR on improving the expression of foreign genes in the nucleus of *C. reinhardtii*.

MATERIALS AND METHODS

Strains and Culture Conditions

Plasmid pKScGFP carrying codon optimized GFP gene was obtained from the Chlamydomonas Resource Center. Plasmid pChlamy3 was obtained from invitrogen for cloning and expression of recombinant protein. The *C. reinhardtii* cell wall-deficient strain *cw15* was purchased from the Chlamydomonas Resource Center. The *C. reinhardtii* strain *cw15* was cultivated in liquid or on solid TAP (Tris/acetate/phosphate) medium at 22-25 °C under a 16 h/8 h day/night cycle (light intensity 50 μE m^{-2} s^{-1}) and on a rotary shaker at 140 rpm. *Cw15* transformants were selected on TAP-agar containing 10 μg ml^{-1} hygromycin. *Escherichia coli* strain TOP10 was grown at 37 °C in Luria Bertani medium. *E. coli* transformants were selected on 100 μg ml^{-1} ampicillin.

PCR and Cloning of GFP in pChlamy Vector

cGFP gene containing 716 bp was amplified from pKScGFP vector using the following primer pairs: Forward primer, containing a restriction site for *Kpn1* (FGFP primer: 5′AGGTACCTATGGCCAAGGGCGAGGAG3′) and the reverse primer with *Not1* restriction site (RGFP primer: 5′ATGCGGCCGCTTACTTGTACAGCTC3′). The PCR conditions consisted of 1 cycle of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C, and a final cycle of 10 min at 72 °C. The cGFP gene was gel extracted by kit (QIAGEN). The PCR product and pChlamy3 were digested with *Kpn1*and *Not1* restriction enzymes and ligated together at a molar ratio of 3:1 insert to vector using T4 DNA ligase. Recombinant pChlamy3-cGFP vector was transformed into *E. coli* TOP10 cells. Afterwards the *E. coli* TOP10 was cultured on Luria-Bertani (LB) agar with 100 μg ml^{-1} ampicillin. The cloning of cGFP into pChlamy vector was confirmed via colony PCR (using FGFP primer and RGFP primer) and restriction endonuclease digestion with *Kpn1*and *Not1*.

Nuclear Transformations

pChlamy3-GFP recombinant plasmid was transformed into cell wall deficient strain of *C. reinhardtii cw15* by electroporation. *C. reinhardtii* was cultured in TAP medium until the culture reached an optical density (750 nm) of 0.3-0.5. Then cells were harvested by centrifugation at 2500 rpm for 10 min at room temperature. Cells were resuspended in TAP containing 40 mM sucrose solution. Next the pchlamy3-GFP vector was linearized by *ScaI* restriction enzyme. The linearized plasmid was electroporated at a voltage of 600 V, capacity of 50 μF and resistance set at infinity (pulse time at -10 ms). Then the transformed cells were resuspended in TAP medium containing sucrose and grown for 24 h under 16 h/8 h day/night light (a light intensity of 50 μE m^{-2} s^{-1}) for recovery. Cells were centrifuged at 2500 rpm for 10 min and resuspended in TAP medium containing sucrose solution before being plated on TAP agar supplemented with hygromycin (10 μg ml^{-1}).

Identification of Gene Positive Transformants by PCR Screening

To investigate the integration of cGFP gene into the *cw15* genome, PCR analysis of cell lysates were performed. Half a colony was resuspended in 10 μl water and heated to 95 °C for 10 min. Then PCR was performed to detect cGFP sequence using the following PCR program: denaturation at 95 °C for 5 min, 35 cycles of: 95 °C for 15 s, 60 °C for 30 s, 68 °C for 1 min and a final extension of 72 °C for 10 min (using FGFP primer and RGFP primer).

GREEN FLUORESCENT PROTEIN ANALYSIS

GFP Analysis by Fluorescence Spectroscopy

The growth graph was drawn to obtain the linear range of growth. To do this, different dilutions of *C. reinhardtii* (1, 1/2, 1/4…) were prepared, and the OD_{750} was measured. Cells were grown in TAP medium containing hygromycin (10 μg ml^{-1}) until the OD_{750} reached 0.8 on a rotary shaker. Cells were harvested by centrifugation at 8000 rpm for 6 min and resuspended in TAP. Then 30μl of cells were transferred into wells of a black 96-well plate. Fluorescence intensity was recorded using a Cytation3, Imaging Reader

Biotek. Wild-type *cw15* strain was used as blank (The process was repeated several times).

Initially, fluorescence excitation and emission spectrum were determined to find the maximum excitation and emission wavelengths, and then the maximum excitation and emission wavelengths were used for analysis of the amount of expressed cGFP in different samples.

Fluorescence Microscopy

Cells were grown in TAP medium with hygromycin (10 μg ml^{-1}) on a rotary shaker until the optical density (750 nm) reached 0.8. Then cells were exposed to excitation at 488 nm and emission at 525 nm.

RESULTS

Amplification of cGFP and Construction of pChlamy3-GFP Recombinant Vector

Specific primers were designed to amplify cGFP from pKScGFP vector. The cGFP PCR product with an expected size of 716 bp is shown in Fig. 1. The PCR product was cloned into pClamy3, and the integrity of the recombinant vector pChlamy3-GFP was confirmed by double digestion using *KpnI* and *NotI* restriction enzymes (Fig. 2) and colony PCR with specific primers.

Nuclear Transformation and PCR Screening of Transformants

The linearized Pchlamy3-GFP vector by enzyme *ScaI* is shown in Fig. 3. The pChlamy3-GFP vector was successfully introduced into the nuclear genome of *C. reinhardtii*. About 100 colonies were clearly observed in TAP medium supplemented with hygromycin (Fig. 4). The integration of cGFP gene in *cw15* genome was confirmed by colony PCR (Fig. 5).

FLUORESCENT ANALYSIS OF TRANSGENIC ALGAE

GFP Analysis by Fluorescence Spectroscopy

To obtain the equal cell counts in all samples, the growth graph should be investigated in linear phase. As shown in Fig. 6, the samples were in linear phase until the OD_{750} reached 0.8.

As shown in Fig. 7a and b, the colonies containing cGFP were examined to identify maximum excitation and emission wavelengths. The cGFP showed a maximum excitation at 488 nm and a maximum emission at 525 nm. 26 colonies of pChlamy3-cGFP were randomly chosen and their fluorescence was measured by a microplate reader. *C. reinhardtii* has endogenous fluorescent pigmants such as flavonoids and chlorophylls that leads to some background. Therefore, colonies were analyzed, by comparing to wild type *cw15*. In some colonies fluorescence higher than the auto-fluorescence was detected (S1-S2-S3-S4) (Fig. 8). S4 colony showed a 28-fold fluorescence more than the auto fluorescence in wild type.

Fluorescence Microscopy

The fluorescence is not observed with a fluorescence imaging system (Axoscope 2 plus fluorescent microscope zeiss Germany).

DISCUSSION

Chlamydomonas reinhardtii is introduced as an important recombinant protein expression system due to being a eukaryote, carrying out post translational modification, ability to export the expressed proteins into the culture medium facilitating its purification [24]. Foreign genes can be introduced into the nuclear genome of *C. reinhardtii* and expressed, although, at a relatively low level. A number of reasons such as epigenetic suppression of gene expression, inefficient transcription (promoter, enhancer element), poor translation of foreign mRNA (codon bias), and positional effects are thought to explain this low expression level [3,6,11,24,25]. Studies have shown that the problem can be solved by using appropriate elements of transcription and translation into the expression cassette of the foreign gene [11,14,15,23,26].

In the present study, *C. reinhardtii* was used as a recombinant protein expression system. At first, cGFP gene was cloned in pChlamy3 vector successfully. Then the recombinant vector (pChlamy-GFP) was transformed into the nucleus of *C. reinhard1tii*. To improve gene expression, a pChlamy3 vector containing hsp70A/rbcs2 chimeric constitutive promoter, hygromycin resistance gene (aph7) for selection in *C. reinhardtii*, a 3′UTR fragment from

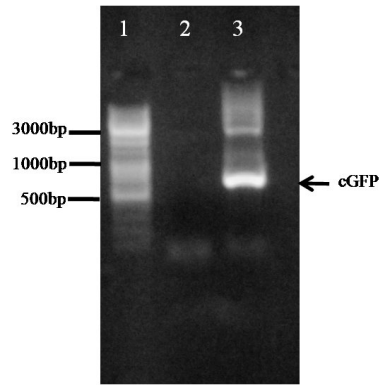

Fig. 1. Electrophoresis of PCR product on agarose gel (1% w/v). Lane 1, DNA ladder mix, lane 2, negative control, lane 3, PCR product (cGFP 716bp).

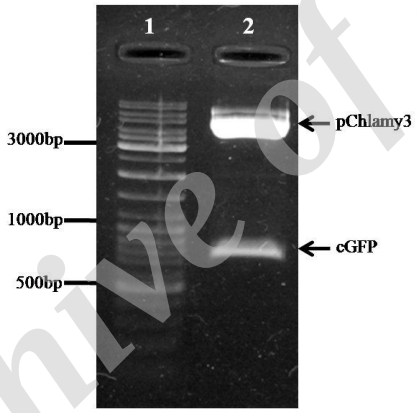

Fig. 2. Confirming of recombinant vector by double digestion. The plasmid was extracted and digested with appropriate restriction enzymes. Lane 1, DNA ladder mix, lane 2, recombinant vector pChlmay3-GFP digested with *KpnI* and *NotI*.

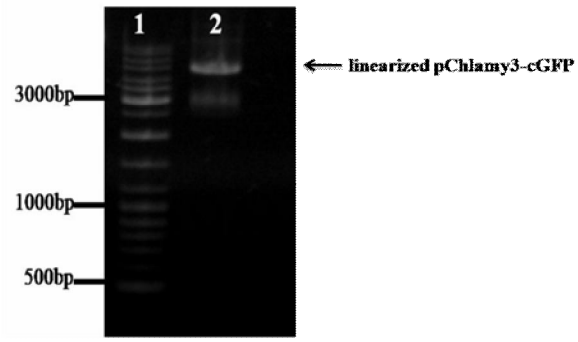

Fig. 3. Preparing recombinant vector for transformation.pChlamy3-GFP vector was linearized by enzyme *ScaI*. Lane 1, DNA ladder Mix, lane 2, linearized pChlamy3-cGFP (approximately 4500bp).

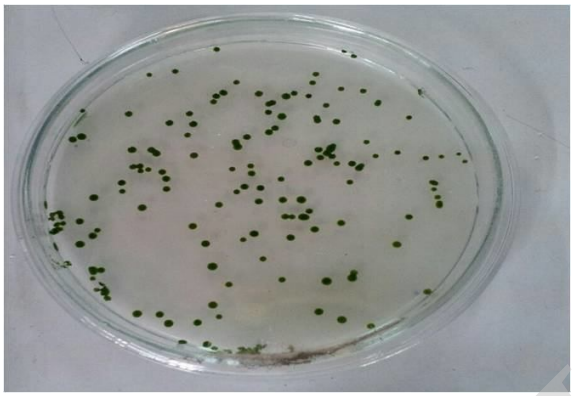

Fig. 4. Colonies were observed in TAP medium supplemented with hygromycin (10 µg ml^{-1}) following transformation.

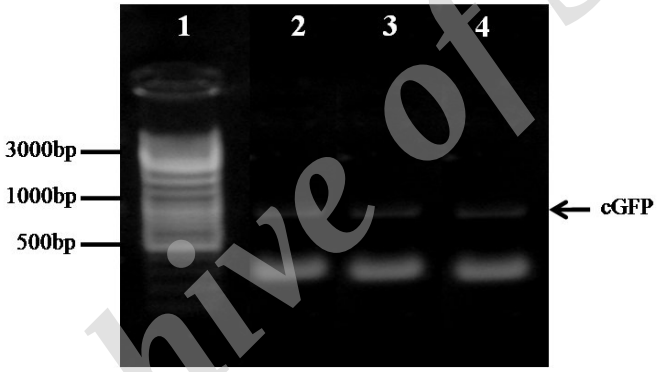

Fig. 5. Electrophoresis of PCR products on agarose gel (1% w/v). The integration of cGFP gene into the genome was determined by PCR analysis of transformed colonies. Lane 1, DNA ladder mix, Lanes 2, 3 and 4 expected band of cGFP (716bp).

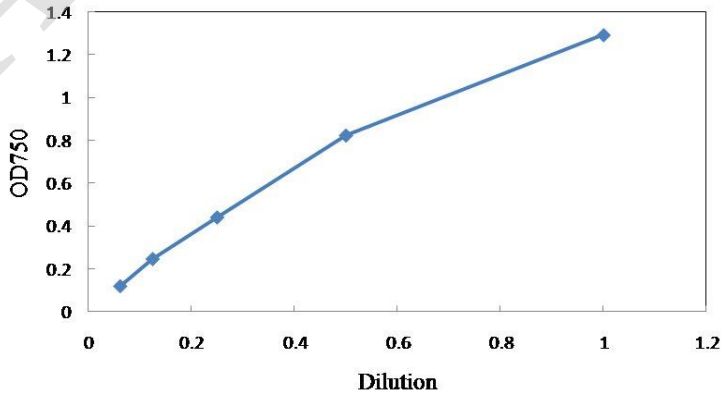

Fig. 6. Growth graph of *Chlamydomonas reinhardtii.*Cells were grown in TAP medium and the OD_{750} of cells were measured by an spectrophotometer.

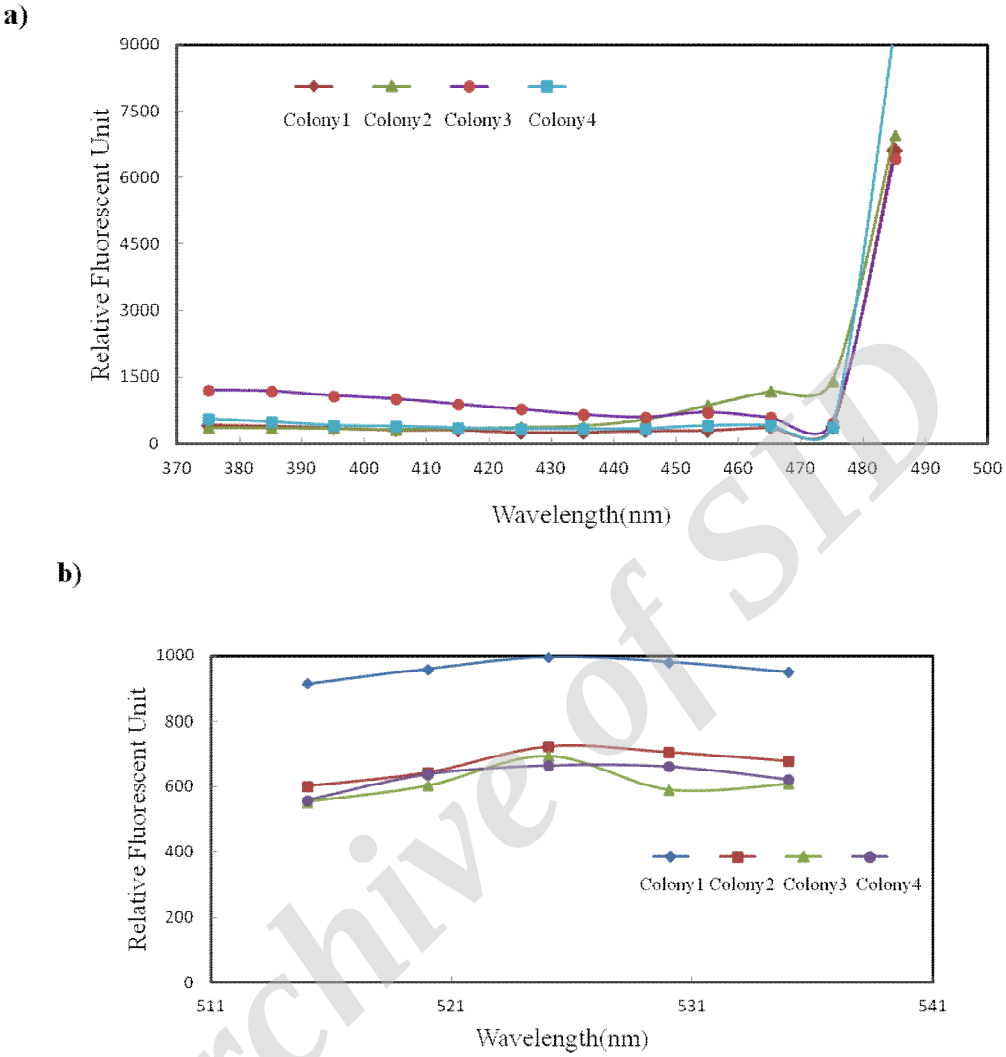

Fig. 7. The 4 clones containing cGFP were examined to determine excitation and emission peaks. As shown, the excitation peak (a) is 488 nm and the emission peak (b) is 525 nm.

rbcs2 (RibuloseBisphosphate Carboxylase/Oxygenase Small Subunit 2), first intron of the small subunit of rbcs2 and ampicillin resistance gene for selection in *E. coli* was used. An appropriate promoter plays a significant role in the expression of foreign genes in *C. reinhardtii* [26]. It has been shown that fusing hsp70A promoter upstream of the rbcs2 promoter results in an intensified promoter activity [12,15]. The transcription terminator also plays crucial roles in gene expression as it determines the [1] stability of a transcript [2], nuclear to cytoplasmic export and [3] translation competence of the transcript [25,27]. So the 3′ UTR fragment from rbcs2 gene downstream of the pChlamy3 multiple cloning site ensures the proper termination of transcript. Also the first intron of rbcs2 has demonstrated to be effective in increasing mRNA and protein accumulation and improving recombinant gene expression [19]. *C. reinhardtii* nuclear genes are highly GC-rich (62%), thus codon optimization is one of the essential steps for increasing protein expression in *C. reinhardtii* [11, 16,28]. In addition, transformation efficiency can increase to 2 fold by plasmid linearization [29]. Hence, pChlamy3-cGFP linearized by *ScaI* was transformed to the nucleus of

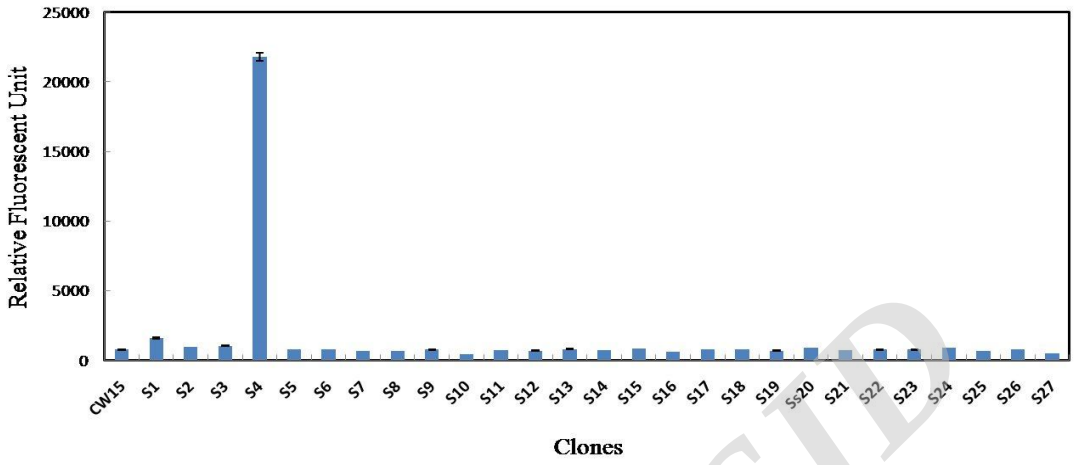

Fig. 8. The relative fluorescence intensity of 27 selected pChlamy3-gfp colonies (S1-S27). The pChlamy3-GFP colonies expressing GFP are shown relative to wild type *cw15*.

C. reinhardtii. Moreover, gene expression in nuclear genome is affected by positional effects due to random integration, and this can change protein expression level in different transgenic lines from the same construct. The number of integrated copies depends on various factors such as cell age, the sequence content, and the size of the gene of interest [26]. Therefore, for foreign gene expression in the nuclear genome of *C. reinhardtii*, screening of colonies to investigate the level of gene expression is required prior to selection of the best transgenic line.

In this study in order to find the best transgenic line, several colonies were screened by PCR to verify the integration of the gene into *C. reinhardtii* genome. Then the fluorescence of positive colonies was investigated by a microplate reader. Among these colonies a colony with a 28-fold fluorescence more than the wild type level was detected, while other colonies showed little increase in fluorescence than the wild type which can be due to random integration and the positional effect.

In previous studies, expression of GFP in the nucleus of *C. reinhardtii* was detected from 1.1 to 3 times more fluorescence than *C.reinhardtii* auto-fluorescence [30,31]. Noor-mohammadi *et al*. used rbcs2, psaD and β2 tubulin promoters and 3´ and 5´ UTR of rbcs2, psaD and β2 tubulin and GFP codon optimization in their studies. Rasala et al. used hsp70/rbcs2 hybrid promoter and introns and 3´UTR of rbcs2 in their studies. Thus it can be concluded that the elements used in this study (*i.e*., hsp70A/ rbcs2 chimeric promoter, a 3′ UTR fragment from rbcs2 and first intron of the small subunit of rbcs2 and codon optimization gene) lead to a greater expression level of the foreign gene.

Results indicate that, applied procedures have successfully improved the gene expression. So we can use this expression system to produce recombinant proteins, in particular, important therapeutic proteins which require post translational modifications. However, due to the positional effect and the random integration, a great number of colonies should be screened to recognize the best expressing transgenic line.

CONCLUSIONS

This study revealed that use of elements including; hsp70A/rbcs2 promoter, first intron of the small subunit of rbcs2, a 3′ UTR fragment from rbcs2 as well as codon optimization lead to improved expression of transgenes in *C. reinhardtii* nucleus.

REFERENCES

[1] E.H. Harris, D.B. Stern, G. Witman, Cambridge Univ. Press, 1989.

[2] O. Pulz, W. Gross, Applied Microbiology and Biotechnology, 2004.

[3] E. Specht, S. Miyake-Stoner, S. Mayfield, Biotechnology Letters, 2010.
[4] T.L. Walker, 2004.
[5] J.P. Davies, A.R. Grossman, Journal of Phycology, 1998.
[6] J. Neupert, D. Karcher, R. Bock, The Plant Journal, 2009.
[7] L. Meslet-Cladiere, O. Vallon, Eukaryotic cell, 2011.
[8] S.S. Merchant, S.E. Prochnik, O. Vallon, E.H. Harris, S.J. Karpowicz, G.B. Witman, A. Terry, A. Salamov, L.K. Fritz-Laylin, L. Maréchal-Drouard, Science, 2007.
[9] C. Remacle, P. Cardol, N. Coosemans, M. Gaisne, N. Bonnefoy, Proceedings of the National Academy of Sciences of the United States of America, 2006.
[10] M. Hippler, K. Redding, J.-D. Rochaix, Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1998.
[11] M. Fuhrmann, W. Oertel, P. Hegemann, The Plant Journal, 1999.
[12] M. Schroda, D. Blöcker, C.F. Beck, The Plant Journal, 2000.
[13] N. Fischer, J.-D. Rochaix, Molecular Genetics and Genomics, 2001.
[14] J. Wu, Z. Hu, C. Wang, S. Li, A. Lei, Chinese Journal of Oceanology and Limnology, 2008.
[15] M. Schroda, C.F. Beck, O. Vallon, The Plant Journal, 2002.
[16] M. Fuhrmann, A. Hausherr, L. Ferbitz, T. Schödl, M. Heitzer, P. Hegemann, Plant Molecular Biology, 2004.
[17] N. Shao, R. Bock, Current Genetics, 2008.
[18] P. Berthold, R. Schmitt, W. Mages, Protist, 2002.
[19] V. Lumbreras, D.R. Stevens, S. Purton, The Plant Journal, 1998.
[20] B.A. Rasala, D.J. Barrera, J. Ng, T.M. Plucinak, J.N. Rosenberg, D.P. Weeks, G.A. Oyler, T.C. Peterson, F. Haerizadeh, S.P. Mayfield, The Plant Journal, 2013
[21] M. Chalfie, Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher, Science, 1994.
[22] R. Heim, A.B. Cubitt, R.Y. Tsien, Nature, 1995.
[23] S. Franklin, B. Ngo, E. Efuet, S.P. Mayfield, The Plant Journal, 2002.
[24] A. Eichler-Stahlberg, W. Weisheit, O. Ruecker, M. Heitzer, Planta, 2009.
[25] A. Kumar, The Ohio State University, 2010.
[26] S. Noor-Mohammadi, 2012.
[27] J. Zhao, L. Hyman, C. Moore, Microbiology and Molecular Biology Reviews, 1999.
[28] M. Heitzer, A. Eckert, M. Fuhrmann, C. Griesbeck, Springer, 2007.
[29] K.L. Kindle, Proceedings of the National Academy of Sciences, 1990.
[30] S. Noor-Mohammadi, A. Pourmir, T.W. Johannes, Biotechnology letters, 2014.
[31] B.A. Rasala, D.J. Barrera, J. Ng, T.M. Plucinak, J.N. Rosenberg, D.P. Weeks, G.A. Oyler, T.C. Peterson, F. Haerizadeh, S.P. Mayfield, The Plant Journal, 2013.