Electroporation-Mediated GFP Gene Transfer into Model Organism
Chlamydomonas reinhardtii

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ABSTRACT: Over the last decade, microalgae has drawn attention as a natural source of valuable compounds and as bioreactors for recombinant protein production. Microalgae-based bioreactor is newly employed for production of safe and cost effective proteins. Especially, *Chlamydomonas reinhardtii*, an unicellular microalga, is the most prominent species which has a short generation time, fast growth rate, multiple genetic systems, and ability to perform posttranslational modifications machinery that plays a significant role in regulating the activity of complex proteins. In this research, the nuclear genome of the *Chlamydomonas reinhardtii* CC-125 strain was transformed by electroporation using construct plasmid pChlamy_3-GFP containing the gene coding for Green Fluorescent Protein (GFP) which is commonly used as an universal marker in biotechnological studies. Molecular and genetic analyses conducted on transformants revealed that the nuclear genome was stably transformed and the transgenes were integrated into the algal chromosomal DNA succesfully, albeit there was no distinct expression level of GFP gene in producing large amounts of protein. Codon optimization, choice of promoters, introns and UTRs, endogenous enhancer elements, regulatory mechanisms, localization of proteins, posttranslational modifications and protease activities are the possible underlying causes of the low expression level.
Key words:*Chlamydomonas reinhardtii,* bioreactor, green fluorescent protein, electroporation

Model Organizma *Chlamydomonas reinhardtii*'ye Elektroporasyon Aracılığıyla GFP Geninin Transferi

ÖZET : Son on yılda, mikroalgler değerli bileşiklerin doğal kaynağı ve rekombinant protein üretimi için biyoreaktör olmaları sebebiyle dikkat çekmektedirler. Güvenli ve ucuz protein üretimi için, mikroalg tabanlı biyoreaktörler yeni yeni kullanılmaktadır. Özellikle de kısa üreme süresine, hızlı büyüme oranına, çoklu genetik sisteme ve kompleks proteinlerin aktivite kazanmaları için gereken post translasyonel modifikasyon mekanizmalarına sahip tek hücreli mikroalg türü *Chlamydomonas reinhardtii* öne çıkmaktadır. Bu çalışmada, biyoteknolojik çalışmalarda evrensel markör olarak kullanılan GFP genini içeren konstrükt pChlamy_3-GFP plazmidi, *Chlamydomonas reinhardtii* CC-125 suşunun nüklear genomuna elektroporasyon yöntemi ile aktarılmıştır. Transformantların moleküler ve genetik analizleri, GFP geninin yüksek miktarda belirgin bir ekspresyon seviyesi olmamasına rağmen nüklear genomun stabil olarak transforme edildiğini, transgenlerin, alg kromozomal DNA'sına başarılı bir şekilde entegre olduğunu göstermiştir. Kodon optimizasyonu, promotör seçimi, intron ve UTR'ler, endojen enhansır elemanları, düzenleyici mekanizmalar, proteinlerin lokalizasyonu, posttranslasyonel modifikasyonlar ve proteaz aktiviteleri, düşük ekspresyon seviyesinin altında yatan muhtemel nedenlerdir.
Anahtar kelimeler: *Chlamydomonas reinhardtii,* biyoreaktör, yeşil floresan protein, elektroporasyon

INTRODUCTION

Natural high-value compounds, especially proteins, in the food, cosmetics, and pharmaceutical industries, are produced from several organisms. Use of proteins provided from natural sources has several limitations, due to the growing world population. With the advent of the recombinant DNA technology, it is possible to clone a piece of DNA or gene of interest and transfer it to another host organism for producing recombinant proteins. Recombinant proteins are considered as a way of providing commercial opportunities to large populations in an inexpensive manner. In various industries therefore it has become inevitable to produce commercial proteins such as vaccines, hormones, enzymes, antibodies, etc. by this way (Griesbeck et al., 2006; Lauersen et al., 2013). Conventionally bacteria, yeasts, mammalian and insect cell cultures or transgenic plants and animals are used as recombinant protein

expression systems (Rasala et al., 2010). In spite of the fact that they have different advantages and disadvantages, the common denominator of all is to produce large amounts of high-quality proteins.

As an alternative to all well known expression systems, microalgae are innovative and feasible eukaryotic host can be genetically modified to produce recombinant proteins. These organisms have been used as a promising source for various industrial and commercial high-value products including fatty acids, pigments, colorants, proteins and carbohydrates providing a broad variety of biotechnological applications range from pharmaceuticals to biofuel (Franklin and Mayfield 2004; Rosenberg et al., 2008; Almaraz-Delgado et al., 2014; Bertalan et al., 2015; Rasala and Mayfield, 2015; Scafie et al., 2015).

More recently biotechnologists pointed out the unicellular *Chlamydomonas reinhardtii* is the most

studied species among the microalgae, that represents a significant contribution to the field of recombinant DNA technology as a bioreactor. It has distinct properties that make it an ideal expression system (Neupert et al., 2012). Firstly, this green algae can be grown rapidly on inexpensive and simple medium of inorganic salts photosynthetically, heterotrophically or mixotrophically and its doubling time is almost 6-8 h (Rochaix, 1995). Apparently they look like microbial cells with their small size that allows for a large surface area-to-volume ratio. Therefore, it can be easily cultured in large quantities in photobioreactors under optimum conditions without transgene escape into the nature or contamination of expressed proteins by viruses, prions or bacterial endotoxins. Furthermore, it is accepted as Generally Recognized as Safe (GRAS) microorganism by the FDA, thus it can be evaluated for oral delivery of therapeutic proteins (Harris, 2009, Specht and Mayfield, 2014).

C. reinhardtii possesses multiple genetic systems located in the nucleus, chloroplast, and mitochondria which have been fully sequenced. In addition *C. reinhardtii* is the only photosynthetic organism whose all three genomes can be genetically altered by well established transformation protocols (Boynton et al., 1988; Kindle et al., 1989; Randolph-Anderson et al., 1993). The vegetative cells of *C. reinhardtii* are haploid, so that if any mutation occurs in the genome, it will be seen immediately on the phenotype (Shimogawara et al., 1997). Even though it is eukaryotic form it can be handled by standard genetic analyses which is similar to bacteria. Lastly, the green algae has ability to perform eukaryotic posttranslational modifications machinery apart from prokaryotic expression systems, which plays a significant role in regulating the activity of complex proteins (Potvin and Zhang, 2010).

All these features mentioned above make this organism an indispensable option for the production of recombinant protein. Here we chose to clone, green fluorescent protein (GFP) whichis released from a kind of jellyfish and commonly used as a reporter gene in biotechnology, cell biology, and biochemistry because it is easy to visualize transgene expression under UV light quickly. Aim of this study is to investigate the potential of introducing reporter gene GFP into the model green alga, *C. reinhardtii*'s nuclear genome by electroporation method to express it.

MATERIALS and METHODS
Strains and growth conditions
The wild type *C. reinhardtii* CC-125 mt+ strain (137c) was obtained from Chlamydomonas Resource Center (St.Paul, MN, USA) and grown photomixotrophically either in liquid or on solid tris-acetate-phosphate medium (TAP) (Invitrogen, USA) under a 12-h light/12-h dark cycle with a light intensity of 155 μmol $m^{-2} s^{-1}$ at 27°C. The liquid culture was shaken manually three to five times.

Escherichia coli strain DH5α was used as a host strain for plasmid construction. Plasmid pETGFP (containing the GFP gene, 5748 bp) was provided by Recombinant DNA Technology Laboratory of Gaziosmanpasa University. Plasmid pChlamy_3 (Invitrogen, USA) was constructed with GFP gene in order to be transferred *C. reinhardtii* nuclear genome. All bacteria strains were grown in Luria Bertani medium (1 % tryptone, 1 % NaCl, 0.5 % yeast extract, pH 7) at 37°C. The LB media for plates were solidified by adding 1% agar that was supplemented with selectable antibiotics.

Plasmid construction
To clone GFP gene into pChlamy_3 plasmid shuttle vector, it was amplified by PCR from plasmid pETGFP using a forward primer (5'-TTTTTTGGTACCATGGGCAGCAGCCATCATCAT -3') and reverse primer (5'-TTTTTTTCTAGATTATTTGTAGAGCTCATCCATG -3') primers.

The PCR products were subjected to restriction enzyme digestion with *Kpn*I and *Xba*I and were ligated into pChlamy_3 plasmid vector that had been digested with the same endonucleases, to generate a recombinant plasmid (pChlamy_3-GFP). Firstly, for plasmid DNA amplification of this final construct (pChlamy_3-GFP), it was transformed into the *Escherichia coli* DH5α strain by heat shock method. Then the cells were spread onto LB agar plates containing 100 μg mL^{-1} ampicillin for the selection of transformants and allowed to incubate for 12-18 h at 37°C. After that succesful transformants were picked and purified using a plasmid DNA purification kit (Biobasic). To make sure that whether DNA fragments were correctly inserted, PCR, restriction digestion and sequencing analyses had been done.

Electrotransformation
We previously linearized pChlamy_3-GFP with *Sca*I enzyme in order to provide more efficient integration into the nuclear genome. Then, we harvested 15 mL of *C. reinhardtii* cells cultured in TAP medium by centrifugation at 2,500 rpm for 10 minutes at room temperature when the optical density of the cell cultures at 750 nm reached between 0.3-0.5. Then pellets resuspended in 250 μL TAP medium supplemented with 40 mM sucrose solution and cells were agitated in the presence of linearized plasmid. After that mixture was incubated for 5 minutes at room temperature in a 4 mm cuvette. Electroporation was carried out using the Eppendorf Eporator at 1190 V pulse length, 2,1 ms width. Electroporated cells were transferred into sterile microtubes and recovered on TAP-40mM sucrose solution for 2 days. After the incubation, the tranformant cells were spread on selection TAP agar plates containing 10 μg mL^{-1} Hygromycin B (hygB) and plates were incubated in a growth chamber at a constant temperature 27°C for 4-8 days.

Genomic DNA isolation,PCR Analysis and Gel Electrophoresis
The hygB resistant transformed colonies were visible after culturing for 4-8 days. The green colonies that

appeared on transformation plates were inoculated into fresh selective liquid TAP to scale up the transformants. The genomic DNA of the transgenic *C. reinhardtii* was isolated from those transformants with the ZymoResearch kit following the manufacturer's protocol. To confirm the integration of GFP gene into the genome, PCR was performed by using the primers GFP *Kpn*I sense and GFP *Xba*I reverse. To observe the product sizes, agarose gel electrophoresis was carried out using 1 % (w/v) agarose gels in 1 X TAE buffer (0.04 M Tris, 1 mM sodium EDTA, 17.5 mM glacial acetic acid) supplemented with 0.1 μg mL^{-1} of ethidium bromide.

SDS-PAGE

100 μl transformant samples of cell culture mixed with 100 μL gel loading buffer and mixtures were denatured at 100 ºC for 2 minutes then loaded 30μL of samples into the each wells of the previously prepared SDS-PAGE gel. With the completion of SDS-PAGE, gel was washed with distilled water, following, stained with Coomassie blue. By means of destaining solution, Coomassie stain was removed from gel. Lastly, using a scanner, the SDS-PAGE gel image was created. (Laemmli, 1970).

RESULTS
Plasmid construction

Amplification of the GFP gene was achieved by PCR using primers designed specific to pETGFP as a template DNA in order clone into shuttle vector pChlamy_3. After digestion of the gene of interest and plasmid vector with same restriction enzymes (*Xba*I-*Kpn*I), purification and ligation, the resulting pChlamy_3-GFP constructsuccessfully created (Figure 1).

To confirm the cloning, restriction enzyme digestion and PCR were performed on positive clones then analyzed with agarose gel electrophoresis. The bands sizes of the vector and insert were respectively 4517 bp, 804 bp as expected (Figure 2).

Following, DNA Sequencing process was carried out with the corresponding PCR primers by Refgen Company (Ankara, Turkey). Analysis of the sequence chromatograms revealed that the cloning was successfully done without any frameshifts.
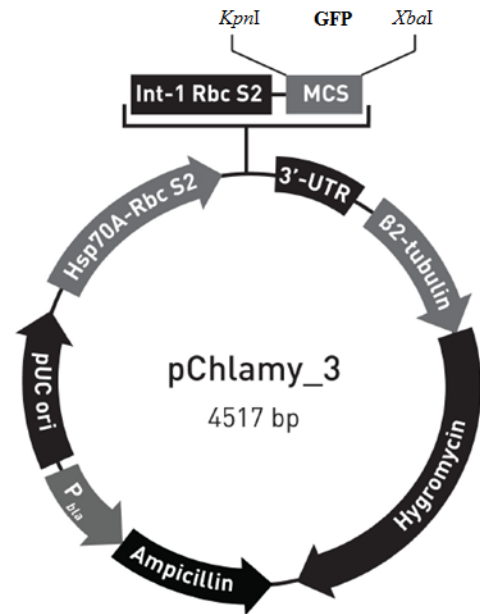

Figure 1. Construction of recombinant plasmid pChlamy_3-GFP. The insert gene (GFP) was cloned into the *Kpn*I and *Xba*I restriction enzyme sites of the pChlamy_3-GFP vector. HSP70/RBCS2, synthetic fusion promoter; 3'UTR, untranslated region; β-tubulin, native promoter from *C. reinhardtii*; Hygromycin, resistance gene; Ampicilin, resistance gene; P$_{bla}$, *bla* promoter, pUC ori, pUC origin of replication (Invitrogen, USA).

Transformation of CC-125 wild type cells of C. reinhardtii

The amplified pChlamy_3-GFP plasmids, positively sequenced, was linearized with endonuclease *Sca*I and inserted into the nuclear genome of the CC-125 wild type cells by electroporation was accomplished with an electric field voltage of 1190 V cm^{-1} and pulse duration of 2.1 ms. Following electroporation, transgenic cells were spread into the TAP medium plates containing 10 μg/mL hygB antibiotic.

After incubation for 4-8 days, when the colonies were screened that exhibited successful transformation, they were streaked onto the new plates (Figure 3).
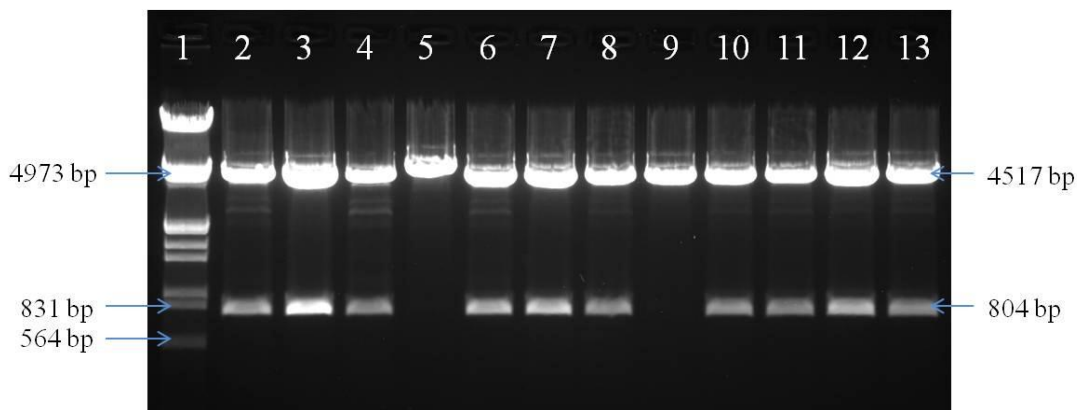

Figure2. It shows the gel electrophoresis results of the DNA digested by restriction enzymes *Kpn*I and *Xba*I in lanes 2, 3,4,6,7,8,10,11,12, and 13 respectively. Lane 5 and 9 are negative ones
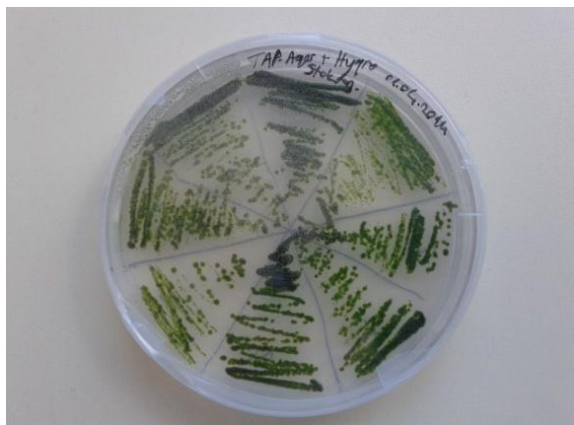
Figure 3. Growth of transformants on the TAP medium.

Molecular analyses of nuclear integration

To verify the nuclear integration of transformants, firstly cells were grown in liquid TAP medium to increase the volume. Genomic DNA isolated from these cells by ZymoResearch kit. Afterwards, PCR was done with designed primers. Gel images of amplicones proved the correct integration (Figure 4).
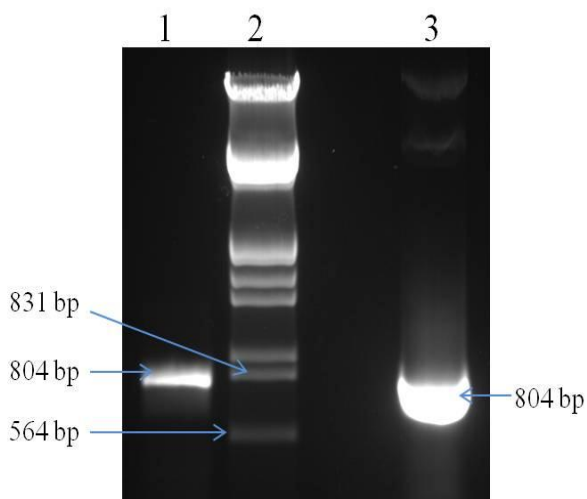

Figure 4. Detection of GFP gene in transformed cells of *C. reinhardtii*. Lane 1, positive control; lane 2, marker; lane 3, transformed cell.

Transgene expression

A SDS-PAGE analysis was done to analyze the GFP protein expression. The protein samples of wild type as a control and transgenic algae were extracted and separated on a 12 % polyacrylamide gel. There was no remarkable band indicating of the GFP accumulation by this method. Further analysis by RT-qPCR and Western blot will be necessary to confirm the presence of transcripts and the recombinant protein expression.

DISCUSSION and CONCLUSION

In this study, GFP was tried to be produced in *C. reinhardtii* by recombinant DNA technology. Our target gene GFP inserted into a plasmid carrying the genes coding for aminoglycoside phosphotransferase (aph7")

as the selectable gene delivered by electroporation into the nuclear genome of unicellular *C. reinhardtii*. Hygromycin resistant transformants isolated by direct selection from the plates. Molecular and genetic analyses carried out on transformants revealed the nuclear genome is stably transformed, however overexpression of GFP couldn't be demonstrated. Accumulation of the desired recombinant protein in *C. reinhardtii* nuclear genome often remains either no or insufficient levels and it's not clear.There are several reasons which may affect the protein synthesis negatively.

One of the underlying causes of it, the lack of codon optimization. Target gene should be compatible with the codon usage of the host organism's genes to enhance expression levels of heterologous proteins (Ferrante et al., 2008). Codon preference of *C. reinhardtii* nuclear genome in the wobble position is approximately %64 GC base pairs. To employ for direct visualizing gene expression and where it occurs, GFP gene should be optimized according to codon usage of the host genome as in previous studies. For instance synthetic eGFP genes with the most frequent codons used instead of rare codons by bioinformatic tools were transferred to *Phaeodactylum tricornutum*. Accumulation of eGFP is 0.3% of total soluble proteins was obtained (Gong et al., 2011). Fuhrmann et al. (1999) also achieved the codon optimized GFP accumulation by nuclear transformation, however, it was low level compared with chloroplast expression of GFP.

Introducing a foreign gene into a host organism's genome may be also extremely important. The integration of a foreign gene into the nuclear genome occurs randomly in any region by non homologous recombination. If the transfer takes place in repeated DNA sequence or heterochromatin gene, it can become directly inactive (Schroda et al., 2002). Algae expression vectors, can be redesigned to ensure integration at any desired locus in the microalgae genome for the higher level of protein production just as in the *Pichia pastoris* yeast system (Balamurugan et al., 2007).

As well as epigenetic changes in gene expression may lead to gene silencing mechanisms. Epigenetic changes such as DNA methylation at the transcriptional, RNA interference (RNAi) at the posttranscriptional level may occur by natural processes. DNA methylation is a system which has capable of inactivating DNA and preventing the protein expression. RNAi inhibits the translation of the specific protein encoded by an mRNA molecule via blocking or degrading (Jeong et al., 2002).

The organization of the chloroplast genome is devoid of complex mechanisms, such as gene silencing due to its similarity to prokaryotic type. Indeed targeted integration of chloroplast organelle by homolog recombination provides respectively high yield of protein (Walker et al., 2005). For this reasons chloroplast genome can be preferred for genetic manipulations instead of the nuclear genome, but unfortunately transformation of this organelle may not be suitable for complex proteins which of them require posttranslational modifications to become an active protein (Mayfield et al., 2007).

Moreover the interaction of the other several factors such as endogenous enhancer elements, regulatory mechanisms, localization of proteins and protease activities, choice of the promoter, introns and UTRs may influence the protein expression in *C. reinhardtii* (Rosales-Mendoza et al., 2012).

All challenges we explained above may cause difficulties in establishing a standard microalgae system to product recombinant proteins. Researchers haven't yet found an effective solution to those handicaps because utilizing of transgenic microalgae in terms of biotechnological studies, is still in the primitive stage (Rasala and Mayfield, 2011). Presently, expressed proteins by this way, have not commercial value on the market to minimize costs, with rates of 0.16-5 % of the total soluble protein (Gong et al., 2011; Kumar et al., 2013).

Future works are needed to remove whole limiting factors from the microalgae expression system and to make it more competitive with the other conventional systems including bacteria, yeasts, mammalian and insect cell cultures, transgenic plants and animals.

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