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Transgenic microalgae as green cell factories for the production of therapeutic proteins

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ABSTRACT

The production of recombinant therapeutic proteins in microbial system has revolutionized biochemistry. The ability to express and purify the desired recombinant protein in a large quantity allows for its biochemical characterization and its use in industrial processes. Poor growth of the host, protein inactivity, and even not obtaining any protein at all are some of the problems often found down the pipeline, where, microalgae can help to solve these issues. Microalgae growth requires inexpensive substrates, thus, these cells can serve as economical and effective bioreactors for obtaining high value-added compounds where low-cost production can greatly impact applicability. During this study, we developed an improved genetic engineering system for the production of therapeutic protein – erythropoietin. The developed genetic transformation system would allow the metabolic engineering and a better alternative to produce recombinant therapeutic proteins from non-model microalgae species.

1. INTRODUCTION

Microalgal transformation is a major contribution in the field of industrial and medical biotechnology, especially for the production of recombinant therapeutic proteins. Gene expression is well established for other microorganisms such as, bacteria, fungi, and yeast; however, the genetic transformation of microalgae is still in its infancy. A number of microalgae genomes have been genetically modified while the

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most frequently reported modifications are in the nuclear and chloroplast genome (Rosenberg et al. 2015; Kumar et al. 2016). The use of microalgae is advantageous as there is no competition with the food crops and the risk of contamination of food resources is low. Additionally, proteins produced by microalgae could be used as orally administered drugs or the whole microalgal cells producing the desired protein could be consumed.

Over the last 2 decades, more than 20 microalgae species have been genetically modified and the recombinant production of therapeutic proteins has been mainly achieved in the chloroplast genome (Gong et al. 2011; Rasala et al. 2014). Despite the advancement in technology, routine transformation is achievable only for a very few species including *Chlamydomonas reinhardtii, Volvox carteri, Haematococcus pluvialis, Phaeodactylum tricornutum*, and some species of *Chlorella* (Bashir et al. 2016). Attempts have been made to produce recombinant erythropoietin (rEPO) from microalgae, but only a minor amount of secreted EPO was obtained from the genetic transformation of *C. reinhardtii* (Eichler-Stahlberg et al. 2009). Thus, there is a need to search an alternative species for the expression and production of recombinant EPO.

The genetic transformation potential of freshwater microalgae species *Dictyosphaerium pulchellum* has not been yet explored with the exception of a previous report by our group (Bashir et al. 2018). Hence, this study investigated the potential of non-model microalgal species as a green cell factory for the production of recombinant erythropoietin protein. During this study, an *Agrobacterium*-mediated genetic engineering system was established for the freshwater eukaryotic microalgae species, *D. pulchellum*, to produce a therapeutic protein - erythropoietin. The T-DNA transfer efficiency is influenced by co-cultivation conditions. Hence, the co-cultivation parameters were also optimized.

2. MATERIALS AND METHODS

2.1 Cultivation of microalgal strains

Economically important freshwater eukaryotic microalgae species *D. pulchellum* was obtained from Korea Marine Microalgae Culture Center, Busan, Republic of Korea. The microalgae species was cultivated in bioreactors in modified-AF6 media at a temperature of 25±2 °C, relative humidity of 50% and a light intensity of 50±2 µmol photons m^{-2} s^{-1}. Growth rates were calculated using spectrophotometer and cell densities were estimated by measuring the dry weight biomass.

2.2 Sensitivity of *D. pulchellum* to selective agents

The *D. pulchellum*'s sensitivity to antibiotics hygromycin (0-100 mg/L) and cefotaxime (0-4000 mg/L) was examined in the liquid and solid modified-AF6 media. The microalgal growth in liquid and solid media was monitored at each alternating day for 15 days and 3 weeks, respectively.

2.3 Construction of expression vector, genetic transformation and stability analyses

The codon optimized human erythropoietin gene (GeneBank accession No. KF178447.1; Bashir et al. 2018) was transferred into the *D. pulchellum* nuclear genome using *Agrobacterium tumefaciens*. Transformed colonies were screened on agar plates containing hygromycin as a screening marker and the transformation efficiency was calculated based on the proportion of the transformed colonies to the number of colonies formed on agar plates without selection pressure. The gene stability and integration analyses were performed through PCR while the erythropoietin expression was estimated by qRT-PCR.

2.4 Purification and quantification of recombinant erythropoietin

The his-tagged recombinant erythropoietin protein was purified from the culture media of the transgenic microalgae through affinity chromatography on Ni-NTA agarose resins (Qiagen, Germany). After dialysis of the isolated protein, it was quantified by BCA protein quantification assay (ThermoFisher Scientific, USA).

2.5 Growth comparison of wild-type and genetically modified *D. pulchellum*

The growth profile of wild-type and genetically modified microalgae species was compared under phototrophic conditions without a selection pressure.

3. RESUTLS AND DISCUSSION

Engineering microalgae has opened a new era for plant biologists and biotechnologists. Genetic transformation of a few industrially important microalgae species such as, *Chlorella vulgaris, C. reinhardtii, H. pluvialis*, has been successful (Stainbrenner and Sandmann, 2006; Rasala et al. 2012; Ahmad et al. 2015), but other promising microalgae still need scientific attention. In our previous study, we reported the microalgae sensitivities to different antibiotics (Bashir and Cho, 2016), approaches to genetic transformation, selectable markers, reporter genes, techniques for genetic modifications (Bashir et al. 2016), as well as successfully produced therapeutic protein from transgenic microalga (Bashir et al. 2018).

To date, more than 30 recombinant therapeutic proteins have been successfully expressed in microalgae, mainly in *C. reinhardtii* (Shamriz and Ofoghi 2017). The unicellular model-alga *C. reinhardtii* is preferred for the expression of recombinant proteins, mainly because of its feasible attributes, such as stable and relatively easy transformation, choice of growth conditions, and availability of various molecular tool kits (Gong et al. 2011). Modifying microalgal genome for the production of extracellular recombinant proteins allows the production of therapeutic proteins independent of the valuable microalgal biomass. However, during the last decade, the use of *C. reinhardtii* has mainly been the focus of research on the production of recombinant proteins. Here, we reported an easier and efficient method for the heterologous expression of recombinant EPO in a non-model freshwater microalga, *D. pulchellum*.

3.1 Antibiotic sensitivity test and genetic transformation

The *D. pulchellum* revealed sensitivity to low concentrations of hygromycin B; it showed sensitivity to hygromycin over 6 mg/L in liquid media and over 10 mg/L on agar plates (data not shown). A normal growth was observed with cefotaxime even at a concentration of 500 mg/L (data not shown). Among the tested acetosyringone concentrations, *D. pulchellum* showed higher transformation efficiency at 150 µM while increasing concentrations of acetosyringone did not further enhance the transformation efficiency (data not shown). The constructed binary vector pCAMBIA-1301-*hyg*-$_{\text{his-tagged}}$*EPO* was electro-transformed into the *A. tumefaciens* strain LBA4404 and the transformation was confirmed through PCR.

The growth and transformation conditions were optimized and the ideal co-cultivation conditions for achieving higher number of hygromycin resistant *D. pulchellum* colonies consisted of a modified-AF6 medium, 150 µM of acetosyringone, 48 h of co-cultivation on agar plates at 25 ± 2 °C, and an 18-h light intensity of 18 ± 2 µmol photons m^{-2} s^{-1} with *Agrobacterium* and *D. pulchellum* cell densities of 1 (OD_{600}) and 1.5 (OD_{750}), respectively.

3.2 Post-transformation analysis

Hygromycin-resistant colonies appeared after 2-3 weeks, and the growth was observed for 6-8 weeks. A higher number of *D. pulchellum* cells in the nonselective medium and no growth of wild-type cells on the selective medium were observed (Fig. 1). The gene stability analysis by PCR showed that the hygromycin-resistant cells retained their resistance phenotype for 24 months and grew well, even when exposed to a higher concentration of hygromycin and showed no loss of hygromycin resistance and retained copies of the *Hyg* gene in their genome. The expected amplicons of 350 bp (Fig. 2a) and 610 bp (Fig. 2b) were observed in the transgenic strains with *EPO* and *Hyg* primers, respectively, whereas, no band was observed in the wild-type and no-DNA template negative controls.

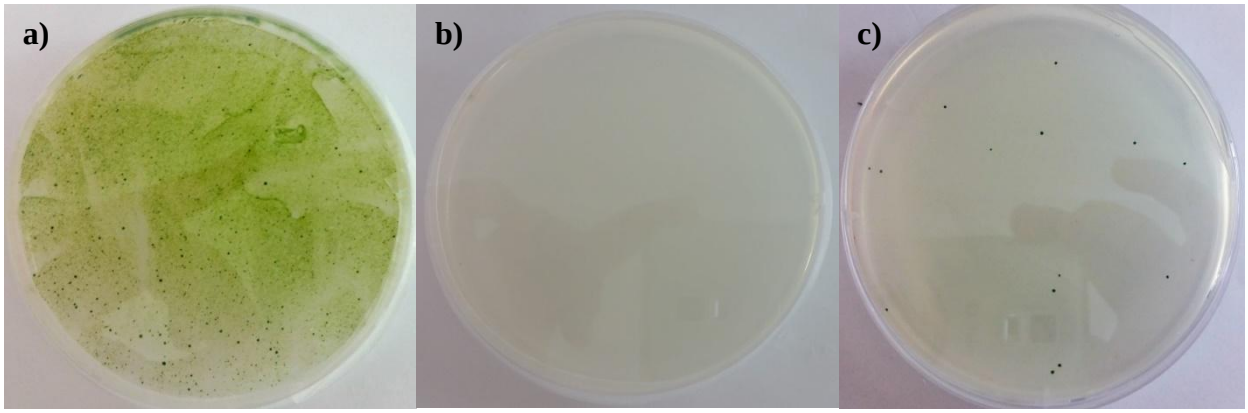

Fig. 1: Wild-type and genetically transformed colonies of *D. pulchellum* (Bashir et al. 2018)

a) Colonies of wild-type *D. pulchellum* on nonselective media, b) Colonies of wild-type *D. pulchellum* on media containing selection markers, c) Colonies of genetically transformed *D. pulchellum* on agar plates containing selection markers

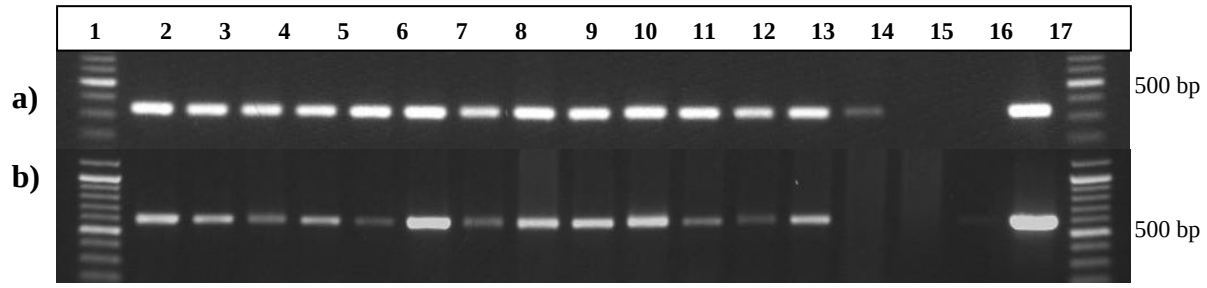

Fig. 2: Gene integration analysis through PCR (Bashir et al. 2018)

PCR amplification of an EPO fragment (a), and a hygromycin gene fragment (b).
Lanes 1 and 19: 10 kb DNA ladder; lanes 2-15: PCR product of transformants; lane 16: PCR product of DNA from wild type as a negative control; lane 17: PCR product of no-DNA template as a negative control; lane 18: PCR product of plasmid DNA as a positive control

3.3 Transgene copy number and erythropoietin expression analysis

Transgene DNA copy number and mRNA expression levels were quantitatively determined using real-time SYBR green qPCR assay. Slightly different EPO expression levels were observed among different transgenic strains and the highest level was observed in the selected strain 2 (Fig. 3). The corresponding T-DNA copy number from qPCR showed no copies in wild-type, whereas, *D. pulchellum* transformed selected strains 1-3 showed copy numbers of 4.88, 6.11, and 4.42, respectively (Fig. 4).

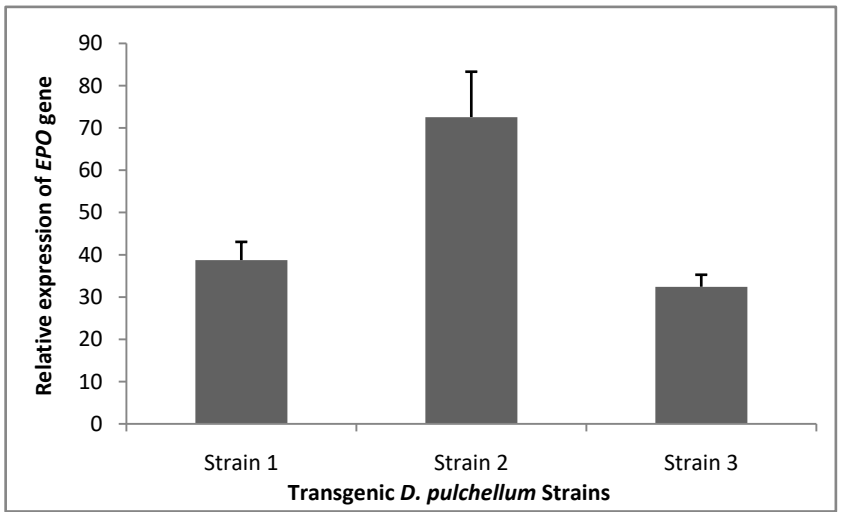

Fig. 3: Relative expression of EPO gene in *D. pulchellum* transgenic strains at 24 months post-transformation (Bashir et al. 2018)

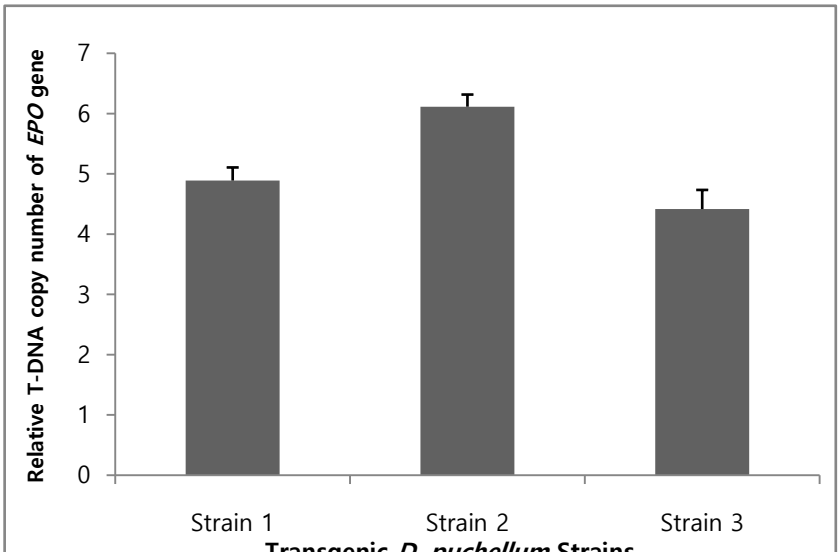

Fig. 4: Relative T-DNA copy number of EPO gene in transgenic *D. pulchellum* strains at 24 months post-transformation (Bashir et al. 2018)

3.4 Growth comparison of wild-type and genetically modified *D. pulchellum*

The growth profile of wild-type and genetically modified *D. pulchellum* was compared under phototrophic conditions. Wild-type strains showed a significantly higher growth than the genetically modified strains (data not shown).

4. CONCLUSIONS

The developed *Agrobacterium*-mediated genetic engineering system was successful and showed an enhanced expression of the recombinant erythropoietin. The simplicity and efficiency of the proposed transformation method allow the use of this system for the expression and production of the recombinant therapeutic proteins from freshwater eukaryotic microalgae. The present study emphasizes that *D. pulchellum* could be used in future as an alternative for the extracellular expression and production of the recombinant proteins.

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