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Designing a new generation of expression toolkits for engineering of green microalgae; robust production of human interleukin-2

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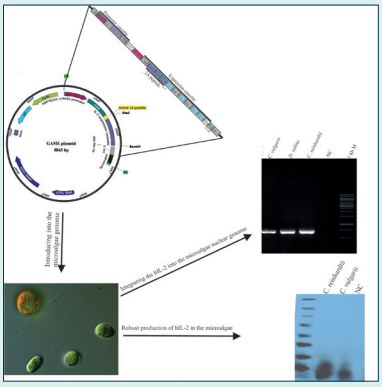
Abstract



Introduction: Attributable to some critical features especially the similarity of the protein synthesis machinery between humans and microalgae, these microorganisms can be utilized for the expression of many recombinant proteins. However, low and unstable gene expression levels prevent the further development of microalgae biotechnology towards protein production.

Methods: Here, we designed a novel "Gained *Agrobacterium*-2A plasmid for microalgae expression" (named GAME plasmid) for the production of the human interleukin-2 using three model microalgae, including *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Dunaliella salina*. The GAME plasmid harbors a native chimeric *hsp70/Int-1/rbcS2* promoter, the microalgae specific Kozak sequence, a novel hybrid 2A peptide, and Int-1 and Int-3 of the *rbcS2* gene in its expression cassette.

Results: The obtained data confirmed that the GAME plasmid can transform the microalgae with high transformation frequency. Molecular and proteomic analyses revealed the stable and robust production of the hIL-2 by the GAME plasmid in the microalgae. According to the densimetric analysis, the microalgae can accumulate the produced protein about 0.94% of the total soluble protein content. The ELISA data confirmed that the produced hIL-2 possesses the same conformation pattern with the acceptable biological activity found naturally in humans.

Conclusion: Most therapeutic proteins need post-translational modifications for their correct conformation, biological function, and half-life. Accordingly, microalgae could be considered as a cost-effective and more powerful platform for the production of a wide range of recombinant proteins such as antibodies, enzymes, hormones, and vaccines.

Introduction

In recent years, much attention has been paid to microalgae in terms of their applications in food, cosmetics, biofuel, and pharmaceutics industries.[1-3] Microalgae are considered as enriched resources of vital compounds such as proteins, antioxidants, unsaturated lipids, and vitamins. As a result, they can be exploited for different biomedical applications even the treatment of various diseases such as cancer.[4] Moreover, the eyespot and channelrhodopsins in the microalgae are located in the center of optogenetic and neuroscience for illuminating the fundamentals of eyes and brain functions.[5-7] Although the genetic manipulation of microalgae is lower than that of the other expression hosts, their key characteristics make them an interesting expression platform for the production of recombinant proteins.[8,9]

They can be used as promising biofactory for the production of a wide range of proteins, in large part because of being "generally regarded as safe" (GRAS) for human usages, the possibility for the scale-up in closed/controlled bioreactors, and the capability to assemble of multi-subunit proteins.[10-12] Nowadays, the potentials of microalgae species such as *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Dunaliella* sp. and *Chlorella*

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vulgaris, have been evaluated for the expression of different type of proteins such as enzymes, hormones, monoclonal antibodies (mAbs) and the other therapeutic biologics.[8,9,13-15]

The transformable compartments of microalga include mitochondria, chloroplast, and nucleus. Of these, the transformations of the two latter organelles have gained more attention for the production of recombinant proteins,[16] while the lack of gene silencing and the possibility of simultaneous insertion of multiple genes are the most important advantages of the chloroplast transformation.[17,18] The chloroplast engineering, however, may face some drawbacks, including the absence of N-glycosylation machinery, intense codon usage bias, and difficulties of the transformation procedures.[19] Altogether, the nuclear transformation seems to provide some benefits such as the capability to perform post-translational modifications such as N-glycosylation and disulfide bridges. Further, it provides a great possibility for the targeting of the expressed proteins into the subcellular compartments or secreting them into the culture media.[8,10] However, the gene expression by the nuclear transformation is low and unstable, in large part because of the use of poor promoters, position effects, transgene silencing, and bias in codon usage.[20] The gene silencing is deemed to happen at both transcriptional and translational levels due to the functional presence of nuclease and proteases in the microalgae cytosol.[4] In addition, the insertion of the genes of interest (GOIs) into the nuclear genome might occur randomly, causing the truncate of the important host genes or even cell death.[10]

Recent researches identified codon optimization using native and chimeric strong promoters and showed that the untranslated regions (UTRs) might be involved in the gene expression of microalgae.[21] Further studies affirmed that the introns (Ints) can impose positive effects on the gene expression levels in eukaryotic cells through the control of mRNA formation and their stabilities and also exporting the transcripts into the nuclear.[22] However, the number, position, and orientation of the inserted Ints in the expression cassette might influence the gene expression efficiency.[23] The 2A peptide is recently utilized to improve the gene expression levels in the different eukaryotic hosts. This peptide consists of ~20 amino acids and involves in the self-cleavage reaction. In fact, during the elongation step of protein translation, a peptide bond could not be formed in the two last amino acids, resulting in the cleavage of the 2A peptide.[24,25] Such peptides were first identified in the foot and mouth disease virus (the so-called P2A), which was then shown to be able to increase the transcription and translation efficiency of the genes.[26] Afterward, several 2A-like peptides (e.g., T2A, E2A, BmCPV 2A and, BmIFV 2A) have been identified in some viruses and named based on their original hosts.[27, 28]

Further, owing to the transferable nature of the T-DNA of the pTi plasmid, the use of *Agrobacterium*-based plasmids is another approach for the stable transformation of microalgae and also a wide range of hosts such as plants, yeasts, fungi, and even human cells.[4,10,29,30]

Herein, we constructed a novel *Agrobacterium*-2A-based (the so-called GMAE plasmid) system, containing *hsp70/Int-1/rbcs2* chimeric promoter, microalgae specific Kozak sequence, a chimeric 2A peptide, Int-1 and -3 of the *rbcS2* gene and UTRs optimized for the robust and stable expression of *hIL-2* gene in the nuclear of the *C. reinhardtii*. The GMAE plasmid is also successfully utilized for the efficient transformation of *C. vulgaris* and *D. salina* cells with the *hIL-2* gene. Molecular and proteomic analyses showed that the GMAE plasmid can stably transform the microalgal cells and robustly produce the hIL-2 protein while the ELISA data confirmed the produced hIL-2 has acceptable biological activity.

Materials and Methods

Materials

The antibiotics (kanamycin, rifampicin, cefotaxime, and chloramphenicol) and also the Ni-Sepharose™ 6 Fast Flow resin were purchased from the Sigma-Aldrich Co. (Missouri, United States). The Taq DNA Polymerase Master Mix for the PCR experiments was obtained from the Ampliqon Co. (Odense, Denmark). The reagents and enzymes used for RNA extraction, cDNA synthesis, and qPCR experiments were purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA). The primary antibodies (i.e., anti-His-tag, anti-hIL-2, and anti-CmR) and also the goat anti-rabbit IgG (alkaline phosphatase conjugate) antibodies, for western blotting, were respectively purchased from the Santa Cruz Biotechnology Co. (Heidelberg, Germany) and Abcam Co. (Cambridge, MA, USA). The reagents for the ELISA assay were obtained from the Bio-Techne Co. (Minnesota, USA).

Microalgae cultivation

The *C. reinhardtii*, *D. salina,* and *C. vulgaris* cells were respectively grown in tris-acetate–phosphate (TAP), modified Johnson's, and BG-11 media. The microalgae were cultured under continuous illumination by white florescent lamps (80 µmol photon m^{-2} s^{-1} irradiance) at 25°C temperature, and 16:8 (light: dark) photoperiod.[10]

Agrobacterium-2A-based plasmid construction

The GMAE plasmid was built on the pCAMBIA1304 backbone. An enhanced *hsp70* and *rbcS2* regulatory elements which are linked through the Int-1 of the *rbcS2* gene were synthesized as a promoter. A novel 5'-UTR, start codon, and microalgae-specific Kozak sequence was placed after the chimeric promoter. Subsequently, the chloramphenicol acetyltransferase (*CmR*) gene, which was used for the selection of the transformed microalgae cells and also the coding sequence of the *hIL-2,* was placed in the construct in the same open reading frame (ORF). Two latter genes were fused together with a novel chimeric

2A peptide. Moreover, two copies of Int-1 were inserted between the *hIL-2* exons. Then, six histidines (i.e., 6x His-tag) were placed before the stop codon, utilizing the hIL-2 purification. It should be noted that the recognizing sequence of the XhoI and BamHI restriction enzymes were respectively inserted before and after the *IL-2* coding sequence for the easy cloning of the other GOIs. Further, one copy of the Int-3 was located after the *hIL-2* coding sequence. The designed construct was synthesized by GenScript Co. and cloned between the SacII and BstEII restriction sites in the pCAMBIA1304 backbone (Figs. S1-S3). The final scheme of the plasmid was achieved by the SnapGene software.

Microalgae transformation procedures

About 200 µL of the microalgae cultures were harvested in the log phase of growth (OD_{660}: 0.4-0.5), cultured on the solidified media and incubated for about five days to form the algal lawns. Then, *A. tumefaciens* strain EHA101, which harbored the GAME plasmid, was incubated at 28°C and 220 rpm overnight in the 2x LB medium containing 20 mg/L rifampicin and 50 mg/L kanamycin. Further, about 350 µL of the *A. tumefaciens* cells were inoculated with 25 mL of the 2x LB medium and incubated at 28°C for about four hours, by which the OD_{660} of the bacterial cells were reached to almost 0.6. Then, the bacterial cells were harvested by centrifugation at 5000 ×g for 5 minutes. The resultant pellets were resuspended in the appropriate liquid media based on the microalgae type. Afterward, about 300 µL of the *Agrobacterium* cultures were spread on the microalgae lawns and co-cultivated at 23°C for 48 hours in the presence of white light. The cocultured cells were harvested and washed three times with the fresh liquid media containing 500 mg/L cefotaxime to eliminate the *Agrobacterium* cells. Finally, the washed microalgae cells were cultured on the solidified media containing the appropriated concentration of the chloramphenicol for the selection of the transformants (Fig. S1).[4, 10]

DNA analysis

Genomic DNAs were extracted at the late log phase of growth from the transformed and untransformed microalgae by using the following buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 1% PVP, 20 mM Na2EDTA, 0.2% LiCl, PH: 8).[31] The presence of the *hIL-2* (i.e., the Exs-3-4) and *CmR* genes was evaluated by polymerase chain reaction (PCR) reactions and the specific primer sets (Table 1).[10] The utilized primer sets were designed by Oligo software version 7 and synthesized by GenScript Company (Nanjing, China). The PCR thermal cycle conditions were initial denaturation at 94˚C for 5 minutes, annealing at 52-62˚C for 1 minute and extension at 72˚C for 1 minute, which was performed for 32 successive cycles followed by a final extension step at 72˚C for 5 minutes (Table 1). The PCR products were analyzed using 1 and 2% agarose gel electrophoresis.

RNA analysis

Total RNAs were extracted from the transformed and untransformed microalgae cells using the TRIzol® reagent according to the manufacture instruction. The quality and quantity of the RNAs were evaluated with a NanoDrop ND®-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis on 2% agarose gel. The complementary DNA (cDNA) from the extracted RNAs were synthesized by the RevertAid First Strand cDNA Synthesis Kit based on the manufacture instruction. The PCR reactions with cDNAs as a template and the specific primers were performed in the same manner mentioned about the DNA analysis. The qPCR reactions were performed to measure the expression level of the *hIL-2* (i.e., the Exs-3-4) gene using a power SYBR™ Green master mix on a Bio-Rad IQ5 real-time PCR detection system (Marnes- La Coquette, France). The α-tubulin gene from the untransformed *C. reinhardtii* is used as a housekeeping control (Table 1).

Western blotting assay

The TSPs were extracted from the transformed and untransformed microalgae cells using the CelLytic™ P Cell Lysis Reagent. The concentration and purity of the extracted proteins were determined by the absorbance at 280 nm in the NanoDrop® ND-1000 spectrophotometer. Further, about 20 mg of the TSPs were subjected to 15% SDS-PAGE gel. After the separation, the proteins were electrophoretically transferred to a nitrocellulose membrane using a semi-dry blotting system (Bio-Rad, Hercules, CA, USA) and blocked with nonfat skim milk

Table 1. The list of used primers

Primers	Forward sequence	Reverse sequence	T_m (C°)
IL-2	5'-ATGGTAGATCTGACTAGTAAAGG-3'	5'-TCATCCATGCCATGTGTAATC-3'	52
CmR	5'-ATGAAAAAGCCTGAACTCACCG-3'	5'-CTATTTCTTTGCCCTCGGACG-3'	56
hsp70	5'-GCAAATTACATATGTCTGCGTG-3'	5'-GTTTACTGACTCTTAAGCTAGCTG-3'	52.5
rbcS2	5'-TCCCATCAAGCTTGCATGCC-3'	5'-AAAGAACATAGGCCCCCTGG-3'	57
Int-1	5'-GTGAGTCGACGAGCAAGCCC-3'	5'- CTGCAAATGGAAACGGCGACG-3'	60
Int-3	5'-GTAAGTCTGGCGAGAGCCCGACG-3'	5'- CTGCGGGCGCACGGGAAATG-3'	62
α-tubulin	5'-CCGCCTGGACCACAAGTTC-3'	5'-TAGTACTCCTCACCCTCGCC-3'	58.5

5% for 24 hours. Furthermore, the membranes were incubated with the primary anti-His-tag, anti-hIL-2, and anti-CmR antibodies (1:1000), and then, the proteins were detected by goat anti-rabbit IgG-alkaline phosphatase conjugate (1:1000) on the basis of the manufacture procedures.

Protein purification and ELISA assay

The TSPs were extracted from 150 mL microalgae cultures to purify the produced hIL-2 protein. Subsequently, the whole extracted proteins were dialyzed through the dialysis tubes with 10 kDa cut-off (Sigma-Aldrich, USA). Further, protein purification was performed by affinity chromatography in which the proteins were incubated with the Ni-Sepharose™ 6 Fast Flow resin at 4°C for 1 hour. After removing the flow-through, the resin was washed two times by binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl and 5mM Imidazole) and subsequently, the 6x His-tagged proteins were collected by elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl and 500 mM Imidazole). The quantity of the purified hIL-2 was evaluated by NanoDrop ND®-1000 spectrophotometer and subsequently, western blotting was performed against the purified hIL-2 by an anti-His-tag antibody.

The ELISA assay was performed for the evaluation of the correct conformation of the microalgae produced hIL-2 compared to that of in human cells. The whole proteins were extracted from the transformed and untransformed *C. reinhardtii* cells using phosphate-buffered saline (PBS) buffer (10 mM Na_2HPO4, 1.8 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl) and sonication and then three concentrations of the extracted proteins (i.e., 10, 20 and 30 µL) were subjected into the ELISA sandwich. Moreover, 10 µL of the total protein from the untransformed microalga was used as a negative control. These approaches were performed three times for each concentration (Table S2). The ELISA analysis was performed by the DuoSet® Human IL-2 kit based on the company instruction.

Statistical analysis

Data were analyzed as the mean values with standard deviation (SD) from three independent experiments. The statistical analyses were utilized by Student *t*-test or ANOVA. A *P* value of less than 0.05 was considered statistically significant.

Results

Designing the GMAE plasmid

To be highly effective on the expression level of genes in the *C. reinhardtii*, the GMAE plasmid was designed as an *Agrobacterium*-2A-based construct consisting of different elements, including *hsp70/rbcS2* promoter, Int-1and Int-3. The *hsp70* (300 bp), *rbcS2* (200 bp) regions and the Int-1 (145 bp) and Int-3 (238 bp) of the *rbcS2* gene were amplified from the *C. reinhardtii* through the specific primers, and subsequently, their sequences were determined (Fig.1 and Figs. S2-S4). The coding sequences of the *CmR* and *hIL-2* were extracted from the NCBI database and then optimized based on the *C. reinhardtii* codon bias. Furthermore, the sequences of the P2A-T2A peptide, in which the coding sequences of GSG amino acids were fused to the C-termini of the peptide. The chimeric promoter (*hsp70/Int-1/rbcS2*) and expression cassettes (*CmR*-2A peptide-hIL-2) and regulatory region (Int-3) were placed continuously in the same ORF. Finally, the 25 kb synthetic construct was cloned into the pCAMBIA1304 backbone through SacII and BStEII restriction enzymes and the GMAE plasmid with 8843 bp size was built (Figs. 1 and 2 and Figs. S1-S3).

Microalgae transformation and its efficiency

Although the GMAE plasmid is optimized for the *C. reinhardtii*, its potential was evaluated for the nuclear transformation of *C. vulgaris* and *D. salina* as well. The chloramphenicol sensitivity assays revealed that 60 and 130 mg/L of the antibiotic were suitable for the transformation of *D. salina* and *C. reinhardtii* or *C. vulgaris*, respectively (Fig. S1). Microalgae transformations were performed via the cocultivation of the *C. reinhardtii*, *C. vulgaris*, and *D. salina* (OD_{660}: 0.5) and EHA101 strain of *A. tumefaciens* which held the GAME plasmid. Further, the microalgae are transferred into the solidified media containing the appropriate concentration of chloramphenicol and after about 10 days the transformed cells appeared in the media (Fig. 3). Our data, based on the number of survived cells on the plates containing chloramphenicol from a count of 10^6 cfu, showed that the GAME plasmid could transform the mentioned microalgae with high frequency. The data

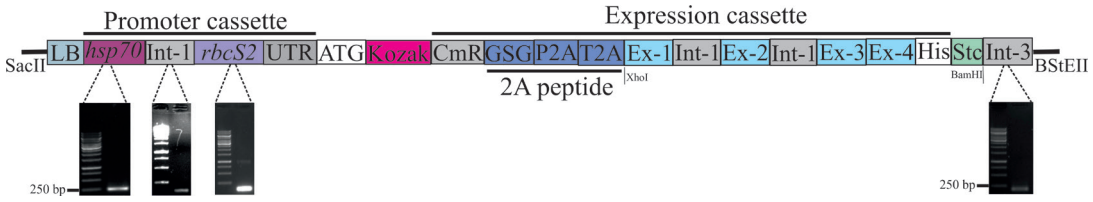

Fig. 1. The schematic image of the deigned construct containing a promoter and expression cassettes. The hsp70, Int-1, rbcS2, and Int-3 elements were isolated and sequenced from the *C. reinhardtii* genome. LB: Left border from the *A. tumefaciens*, Ints: Intron 1 of the rbcS2 gene, *CmR*: Chloramphenicol acetyltransferase, Stc: Stop codon (i.e., TAA). The synthetic elements were cloned into the pCAMBIA1304 backbone through the SacII and BStEII restriction enzymes.

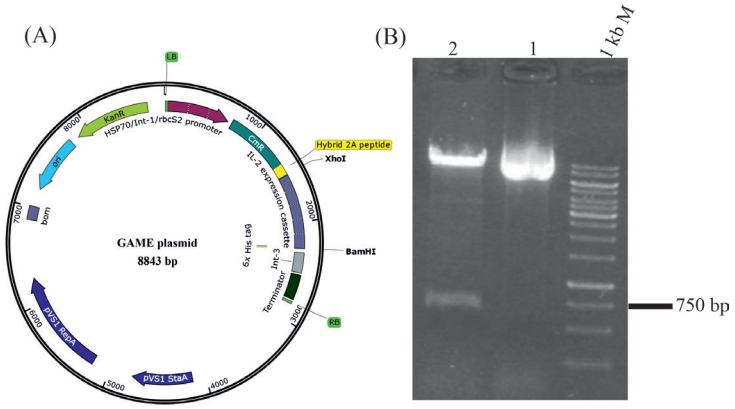

Fig. 2. The structure of the GMAE plasmid and its electrophoresis. (A) The plasmid is an *Agrobacterium*-2A-mediated construct optimized based on the *C. reinhardtii* codon bias. (B) The size of the GMAE plasmid is 8843 bp (Lan 1) according to electrophoresis analysis. For more validation about the construction of GMAE plasmid, the vector was double digested with BamHI and XhoI restriction enzymes with restriction sites at the first and end of the hIL-2 expression cassette. As expected, two bands with ~760 bp and ~8 kb were determined after electrophoresis (Lan 2).

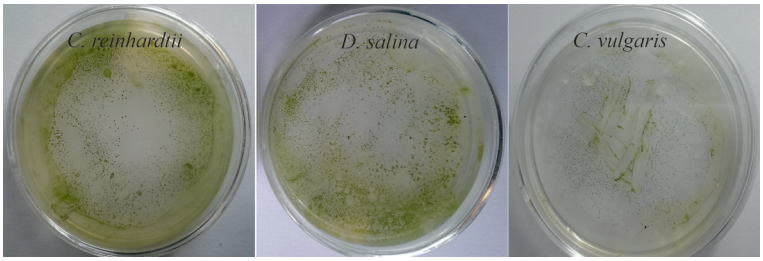

Fig. 3. Appearing the chloramphenicol resistance microalgae cells in the solid media containing the appropriate concentration of the antibiotic**.** The transformation frequency in the *C. reinhardtii* is relatively higher than the *D. salina* and *C. vulgaris*.

revealed the transformation efficiency is relatively higher in the *C. reinhardtii* (157 ± 0.8 cfu per 10^6 cells) compared to the *C. vulgaris* (157 ± 0.8 cfu per 10^6 cells), and *D. salina* (157 ± 0.8 cfu per 10^6 cells), as shown in Fig. 3 and Table 2.

T-DNA integration into the microalgae genomes

The PCR experiments from the genomic DNA of the transformed *C. reinhardtii*, *C. vulgaris*, and *D. salina* with the specific primers (i.e., ~654 bp and ~252 bp amplicons as shown in Table 1) confirmed the successful integration of the Cm^R and *hIL-2* (i.e., the Exs-3-4) genes into the microalgae genomes respectively (Figs. 4A and 4C). Further, the PCR reactions by the primers (Table 1) and the cDNAs from the transformed and untransformed microalgae as template validated the same results, indicating the successful transcription of the genes (Figs. 4B and 4D). Furthermore, the detection of the *hIL-2* was reconfirmed by the qPCR, in which the results showed the expression level of the *hIL-2* is stronger in the *C. reinhardtii* in comparison with those of the *C. vulgaris*, and *D. salina* (Fig. 5C).

Production of hIL-2 protein in the microalgae

Western blotting data identified a 15 kDa sharp band in the transformed *C. reinhardtii*, *D. salina*, and *C. vulgaris* (Fig. 6A). The data showed an additional band with a size of ~34 kDa is presented in the transformed *C. vulgaris*, and *D. salina* cells, which appeared to be the dimerized hIL-2 proteins (Fig. 6A). Interestingly, semi-quantitative data revealed that the expression levels of hIL-2 displayed no significant differences in the transformants, in large part because of the possible similarity in the codon bias of *C. reinhardtii*, *C. vulgaris*, and *D. salina*.

Functional analysis of the 2A peptide in the microalgae

Regarding the self-cleaving characteristic of the 2A peptides, the correct processing of the expression cassette (i.e., *CmR-2A-hIL-2*) during the translation could make

Table 2. Transformation frequency by GMAE plasmid

Microalgae	No. of cells used for transformation	No. of transformants	Transformation frequency (cfu/10^6 cells)
C. reinhardtii	4.6×10^6	815	177 ± 1.7
D. salina	5.1×10^6	758	148 ± 6.2
C. vulgaris	4.8×10^6	563	117 ± 2.9

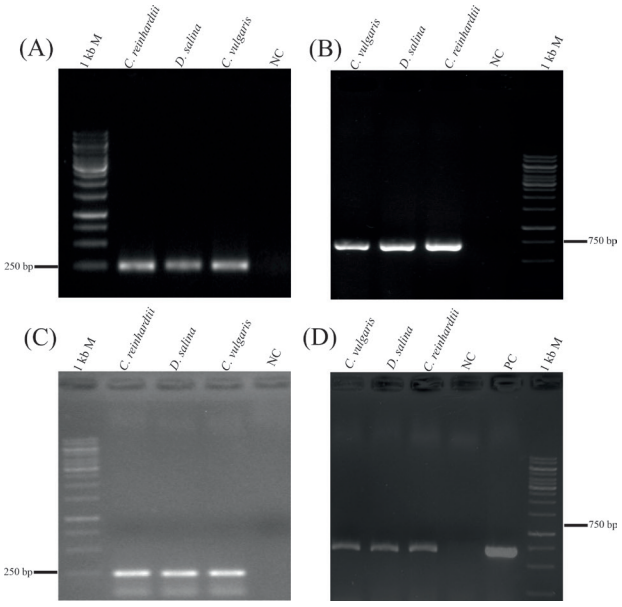

Fig. 4. Successful integration and transcription of the *IL-2* (panels A and C) and *CmR* (panels B and D) genes into the genome and transcriptome of the *C. reinhardtii*, *D. salina*, and *C. vulgaris*. NC: negative control (i.e., untransformed *C. reinhardtii*), PC: Positive control (GMAE plasmid).

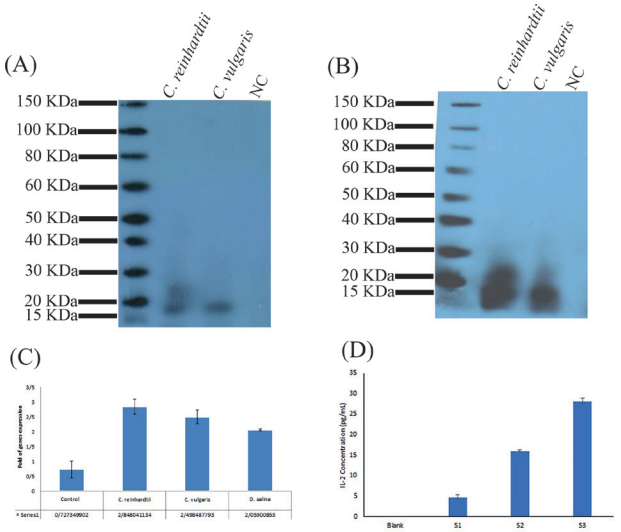

Fig. 5. Stable hIL-2 expression, production, purification, and biological activity assays. (A) After five months from the transformation, western blotting with the anti-His-tag antibody confirmed the presence of hIL-2 in the transformed *C. reinhardtii*. (B) Successful purification of hIL-2 from 150 mL culture of the *C. reinhardtii* cells. Densitometry analysis of western blotting bands by the ImageJ software identified the hIL-2 can accumulate approximately 0.94% and 0.59% of the total soluble proteins of the *C. reinhardtii* and *C. vulgaris* respectively. (C) The qPCR data identified the expression level of *hIL-2* in the transformed *C. reinhardtii* is somewhat higher than the *C. vulgaris* and *D. salina* cells. (D) The ELISA analysis of the whole extracted proteins from the transformed *C. reinhardtii* revealed the acceptable biological activity of the expressed hIL-2. Control: α-tubulin gene of the untransformed *C. reinhardtii*, S1, 2 and 3: Different elution of the whole extracted proteins from the transformed *C. reinhardtii*, Blank: whole extracted proteins from the untransformed *C. reinhardtii*.

CmR and hIL-2 as the discrete proteins. The screening of chloramphenicol resistance colonies in the *C. reinhardtii*, *D. salina*, and *C. vulgaris* could be the first evidence of the proper processing of the 2A peptide (i.e., GSG-P2A-T2A) in the microalgae (Fig. 3). Moreover, western blotting with anti-His-tag and anti-CmR antibodies showed the mentioned microalgae can completely process the 2A peptide in which ~15 (related to the hIL-2) and ~26 kDa (related to the CmR) distinct bands could determine in the TSPs of the transformants. Moreover, the unprocessed CmR-2A-hIL-2 protein, which has a molecular weight of about 41 kDa, was not identified during the immunoblotting analyses (Figs. 6B and 6C).

Protein expression stability and quantity

The stability and quantity of the expressed hIL-2 are assessed in the extracted proteins of the transformed *C. reinhardtii* and *D. salina* after 5 months from the transformation. The western blotting analyses represented that the transformed colonies could stably produce the hIL-2 protein in similar concentration to the freshly transformed microalgae (Fig. 5A).

Protein purification and ELISA assay

The produced hIL-2 in the *C. reinhardtii* and *C. vulgaris* was extracted and purified from the 150 mL of the microalgae culture in the late exponential phase of growth through 6x His-tag. Western blotting with anti-hIL-2 antibody confirmed the robust quality and quantity of the produced protein (Fig. 5B). The semi-quantitative analyses from the TSPs and purified hIL-2 revealed that the transformed *C. reinhardtii* and *C. vulgaris* could respectively produce about 0.94% and 0.59% of the recombinant protein (Figs. 6A and 5B). Furthermore, the ELISA analysis confirmed the *C. reinhardtii* produced IL-2 with proper conformation compared to that of the human cells. Based on these findings, the produced IL-2 showed acceptable biological activity (Fig. 5D, Fig. S5 and Table S1).

Discussion

Most of the currently approved/used biologics are complex proteins, which contain glycosylation sites and disulfide bridges required for their correct conformation, biological functions and pharmacokinetic and pharmacodynamic properties [32]. The hIL-2 protein harbors one glycosylation site in the Thr-3 and two disulfide bridges in the 58 and 105 amino acid residues. As a vital secreted cytokine, hIL-2 plays a fundamental role in the regulation and activation of different immune cascades.[33] This protein is routinely used for the immunotherapy of various malignancies, including kidney, liver, gastric, and colorectal cancers.[34] Besides, hIL-2 could be utilized as a strong adjuvant for the deigning of various human and animal vaccines.[35] Currently, the recombinant hIL-2 is produced commercially in the *Escherichia coli* cells (Aldesleukin™ or Proleukin™ marketed by Prometheus Laboratories Inc.), which are a non-glycosylated molecule. Of note, the glycosylation is necessary not only for the biological

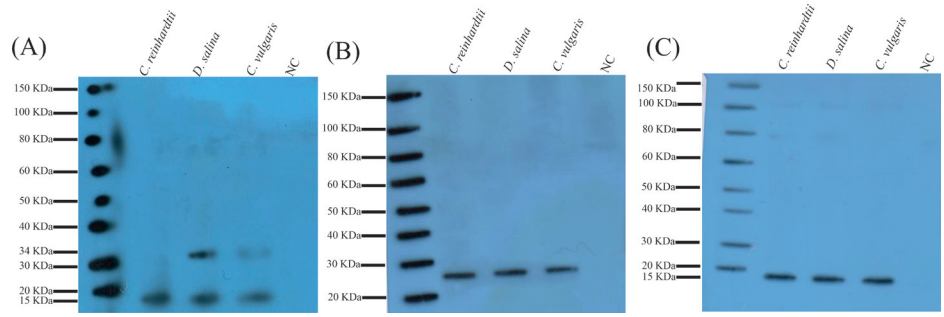

Fig. 6. The western blotting analyses of the transformed microalgae cells. (A) Detection of hIL-2 with the size of 15 kDa (similar to the human IL-2) in the *C. reinhardtii*, *D. salina*, and *C. vulgaris*. The additional 34 kDa band shows the dimerized IL-2 in the *D. salina* and *C. vulgaris*. Panels B and C represent the western blotting analyses with the anti-*CmR* and anti-His-tag antibodies respectively. These data show that the transformed microalgae can entirely process the GSG-P2A-T2A self-cleaving peptides.

activity of hIL-2 but also for increasing the solubility and *in vivo* half-life of this protein.[36] Further, the lower solubility of the non-glycosylated hIL-2 causes somewhat hurdles in terms of the production, formulation, and administration. Moreover, the formation of insoluble inclusion bodies during *E. coli* protein expression is another important problem, upon which hIL-2 protein may aggregate and show immunogenicity to some extent.[37] In this sense, exploring a novel powerful expression system is highly demanded for the efficient production of various biotech drugs.

Microalgae are considered as an alternative expression platform for the production of a wide range of different biologics, in large part because of some critical features such as showing great resilience in the production of glycosylated proteins, and similarity of the protein synthesis machinery to that of the human.[4, 10] However, the main bottleneck of the microalgae transformation is the transient and poor expression of GOIs from the nuclear genome which may limit its usefulness for the production of protein-based pharmaceuticals.[8] To tackle these challenges, we described a novel approach for the achievement of stable and high expression levels of recombinant proteins during the nuclear transformation. We previously reported the usefulness of the pCAMBIA1304, as an *Agrobacterium*-based plasmid and showed the stable expression of the green fluorescent protein and β-glucuronidase as a reporter (*gfp:gus*) fused genes in the green microalga *Dunaliella pseudosalina*.[10] Further, we have implemented the same approach for the stable and efficient expression of the *gfp:gus* fused genes in the blue-green microalga *Spirulina platensis*, which contains more than ten resident restriction enzymes.[4] Similarly, the LBA4404 strain of *A. tumefaciens* is utilized to stably transform the pCAMBIA1304 into the genome of *C. reinhardtii*.[38] The stable expression of enhanced *gfp:gus* genes in the *Schizochytrium* protoplasts has already been reported in *A. tumefaciens* strain EHA105 and LBA4404, where the latter strain displayed the higher transformation capacity.[39] Of note, the *A. tumefaciens* strains possess a group of genes (*VirD2*, *VirE2*, *VirE3,* and *VirF*), whose products are effective on the correct and efficient nuclear transformation. Indeed, VirD2 protein is linked to the 5′ end and also the VirE2 is covered the whole length of the GOI, which can protect the GOI from the inadvertent degradation by the host endonucleases.[10] After importing the gene into the host cell, the nuclear localization signal existed in the VirD2 and VirE2 proteins and also VirE2 help the gene to direct into the nucleus. In the nucleus, the VirF protein mediates the uncoating process in which the gene is integrated into the transcriptionally active regions of the host genome.[40] Accordingly, we built our expression cassette into the pCAMBIA1304 backbone (Fig. 1) to achieve the *Agrobacterium*-based expression system (Fig. 2), which resulted in a specific transformation of the microalgae with high efficiency and stability (Figs. 3, 4 and 5). In this line, we used the *hsp70* with an additional 3'-UTRs and *rscS2* promoters that fused together through the Int-1 of *rscS2* gene. Moreover, an enhanced 5'-UTR and the microalgae specific Kozak sequence were added in the expression cassette, in which the position of start codon is presented for the first time in the microalgae transformation procedures. Because of the regulatory nature of the Int-3 from the *rscS2* gene in the transcriptional and post-transcriptional levels, the element was inserted in the downstream of the expression cassette (Figs. S1-S4). Further, the codon usage optimization of the heterologous genes and use of the strong native promoters or chimeric constitutive promoters (e.g., *hsp70/rbcS2*) could elicit a significant increase in the level of expressed genes.[41, 42]

Besides, consistent with our results, codon-optimized hepatitis B surface antigen (HBsAg) and its corresponding Ab have been expressed in a high expression level in the microalga *P. tricornutum* using nitrate reductase (*NR*) promoter and terminator.[42] Additionally, the existing of UTRs (e.g., 3'-UTRs) on the downstream of heterologous genes is effective on the gene expression efficiency. The 3'-UTRs contain elements, which are involved in the correct termination of the transcripts via regulation translation efficiency, the stability of mRNA, and polyadenylation signals.[43, 44] Surprisingly, it seems the first intron of *rbcS2* gene in *C. reinhardtii* and also intron 1 or introns 9 and

10 of *NR* gene in *Volvox carteri* could improve the post-transcriptional and post-transitional expression levels of GOI.[45, 46] Using the 2A peptides in the expression cassette is another trend to increase the quantity and stability of microalgae expressed genes. It is noteworthy that the linking of the P2A peptide to GFP was shown to significantly increase the fluorescent density during the nuclear expression of the *C. reinhardtii*.[8] In addition, the peptide is inserted between *sh-ble* gene and the coding sequence of the xylanase I (Xyn1), as a key industrial enzyme. This method could highly improve the accumulation of the protein (i.e., almost 100 times) compared to unlinked Xyn1 expression.[16] Some previous reports showed that the cleavage efficiency of T2A-P2A was higher overusing solely of the peptides in the eukaryotic organisms.[28] Moreover, it is identified that the presence of the glycine-serine-glycine (GSG) spacer on the N-termini of the 2A peptides could highly increase the cleavage efficiency in the expression hosts.[27] Accordingly, we used a chimeric 2A peptide (i.e., GSG-T2A-P2A), which was inserted between the *CmR* and coding sequences of *hIL-2* genes (Figs. 1 and S3). There is only one report about the gene expression via 2A peptides in microalgae, in which the P2A peptide was specifically used for the expression of GFP and Xyn1 proteins. However, the microalga could not entirely process the P2A peptide, in which the un-cleaved proteins (i.e., ble-P2A-GFP and ble-P2A-Xyl1) were produced in the *C. reinhardtii* cytosol.[8] Previously, the heavy and light chains of a human monoclonal antibody (i.e., GL4mAb) were expressed in *P. tricornutum*, with results showing the transformed microalga could robustly produce the recombinant mAb.[42] Moreover, in another study, the stable expression of Xyn1 in the *C. reinhardtii* revealed the microalga can accumulate the produced protein about 0.25% of the TSP.[8] Our data, however, showed that the used chimeric 2A peptide could entirely proceed in the *C. reinhardtii*, *C. vulgaris*, and *D. salina* and no additional un-cleaved protein (i.e., CmR-chimeric 2A-hIL-2) was found to be exhibited in their cytoplasmic proteins (Fig. 6). Moreover, our results showed that the GMAE plasmid could stably transform the *C. reinhardtii*, in which the produced IL-2 accumulated about 0.94% of TSP (Fig. 5).

To the best of our knowledge, the GMAE plasmid can reach the highest level of expression in the nuclear transgenes in the microalgae. Collectively, this study is the first successful attempt in the microalgae towards the production of biologics by the proposed strategy using the GAME plasmid.

Conclusion

The major limitations of different expression systems (e.g., bacteria, yeasts, plant, and mammalian cells) and the tempting features of the microalgae (e.g., glycosylation similarity to the human) have inspired many scientists to capitalize on the microalgae for the production of recombinant proteins. However, the transient and low gene expression seems to be the two important bottlenecks during microalgae transformation which limited microalgae-based recombinant protein production. Here, for the first time, we showed that the engineered GAME plasmid system could result in stable and robust production of IL-2 in three microalgae, including *C. reinhardtii*, *C. vulgaris*, and *D. salina*. The GAME plasmid system can indeed pave the way towards the stable microalgae-based production of biomacromolecules such as IL-2 with appropriate post-translational modifications. Despite being able to produce IL-2 in the microalgae (i.e., in *C. reinhardtii*, *C. vulgaris*, and *D. salina*), we believe that the functionality of the produced protein should be validated in an animal model before its translation into clinical applications. Further, the large-scale production of any biologics in this host demands further optimization for the industrial scale. Taken all, for the first time, we introduce the proposed GAME plasmid system that can be used for the high yield production of biologicals with excellent stability in microalgae.

Research Highlights

What is the current knowledge?

√ Microalgae can consider as a more powerful platform for the production of different types of therapeutic proteins such as hIL-2.

√ Because of the poor and unstable gene expression in the microalgae, exploring the new approaches for solving these limitations have intensely demanded.

What is new here?

√ A novel *Agrobacterium*-2A-based plasmid (so-called GAME) was constructed for the engineering green microalgae to robustly producing the human IL-2 (hIL-2).

√ The engineered microalgae can stably accumulate the hIL-2 about 0.94% of the total soluble protein with acceptable biological activity.

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Ethical statement

This study was approved by the ethical committee of Tabriz University of Medical Sciences (Ethics No. IR.TBZMED.REC.1396.781).

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

All authors conceived and planned the project. JD performed the experiments. JD wrote the draft and KA, and AM finalized the latest version of the manuscript. All authors contributed to discussing the results.

Supplementary Materials

Supplementary file 1 contains Figs. S1-S5 and Tables S1-S2.

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