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Evaluating nuclear transgene expression systems in *Chlamydomonas reinhardtii*

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ABSTRACT

Chlamydomonas offers several potential advantages as a single-celled autotrophic recombinant protein production system including: facile transformation systems for all three genomes, low production costs, and the ability to secrete proteins. However, transgene expression levels from the nuclear genome of *Chlamydomonas* are often inadequate for industrial applications. With the objective of optimizing nuclear transgene expression, we surveyed transgene (luciferase) expression driven by seven different nuclear gene promoters and three different transcription terminators. The results demonstrate that in addition to the choice of nuclear gene promoters used, transcriptional terminators can have strong influence on transgene expression. We show that the *psaD* terminator improved transgene expression when paired with a variety of different gene promoters. Among those tested, the *psaD*–*psaD* promoter–terminator expression cassette gave the highest expression levels. This expression cassette was then used to express a human protein of pharmaceutical value, human butyrylcholinesterase (huBuChE). *Chlamydomonas* cells were able to accumulate the luciferase–huBuChE fusion protein to 0.4% of total soluble protein levels, which is comparable to the expression levels of the same protein obtained in plants. In addition, this study found that the form (linear or supercoiled) of the transforming DNA used for *Chlamydomonas* transformation had significant impact on the transformation efficiency and the level of transgene expression and stability. These results demonstrate that transgene expression in *Chlamydomonas* can potentially be scaled for commercial production of recombinant proteins.

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1. Introduction

The unicellular microalga *Chlamydomonas reinhardtii* has become the organism of choice for studying photosynthesis, flagellar structure and assembly, organelle biogenesis and cell–cell interactions during mating [1]. *Chlamydomonas* can reproduce sexually or asexually and can grow either photoautotrophically, heterotrophically and/or mixotrophically. The availability of a complete genome sequence, a large collection of mutants, coupled with the ability to manipulate all three genomes (nuclear, chloroplast and mitochondrial), along with its well characterized genetics have generated a lot of interest in the biotechnological applications of *Chlamydomonas* [2–5]. The potential biotechnological applications of *Chlamydomonas* range from the production of biofuels, hydrogen, and recombinant proteins, to biopharmaceuticals.

Even though *Chlamydomonas* can be genetically engineered to express recombinant proteins from either the nuclear, chloroplast or mitochondrial genome, protein production from the single large chloroplast of *Chlamydomonas* has generated the greatest yields to date [6,7]. The ability to direct the site of transgene integration into the chloroplast genome through homologous recombination and the lack of mechanisms for transcriptional and post-transcriptional gene silencing in the chloroplast all contribute towards stable and often excellent levels of transgene expression [6–8]. While proper folding of many recombinant proteins and the formation of disulphide bonds have been reported in chloroplasts [6,8–12], chloroplasts lack the enzymes and machinery required to carry out other important post-translational modification of proteins such as glycosylation, which contributes to stability and often functionality of a protein. Thus, the inability of chloroplasts to produce glycosylated proteins is a major drawback of recombinant protein production from chloroplast. In addition, the protein processing and targeting machinery necessary for secreting recombinant proteins into the culture media is only operational for nuclear encoded proteins.

Recombinant protein production from the nuclear genome of *Chlamydomonas* can overcome the limitations of post-translational modification and processing associated with chloroplast expression.

Abbreviations: CVDP, *Chlorella* virus DNA polymerase promoter; RLU, Relative luminescence units.

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Nuclear transformation of *Chlamydomonas* can be easily performed using particle bombardment [3,13], glass beads [14], electroporation [15] or silicon whiskers [16]. Moreover, the use of mutant strains lacking intact cell walls permits secretion of the recombinant protein into the culture media allowing easy protein purification thereby further reducing the cost of recombinant protein production. Nuclear expression also offers the flexibility of targeting, attaching and displaying the recombinant proteins on cell membrane, where the recombinant protein can function as an immunogen inducing an immune response and serving as a vaccine [7,17]. However, poor expression of transgenes from the nuclear genome of *Chlamydomonas* has been the major impediment in developing *Chlamydomonas* as a biofactory for production of recombinant proteins. Currently, the molecular mechanisms limiting nuclear transgene expression are not well known. Possible mechanisms for low nuclear-encoded protein expression include gene silencing, inefficient transcription from heterologous promoters, improper RNA processing, mRNA instability, and instability of protein products [18–20]. Although significant improvements have been made in transgene expression by using improved *Chlamydomonas* gene promoters [18,21–24], codon optimization of transgenes [25], and inclusion of introns [22,24], transgene expression from the nuclear genome of *Chlamydomonas* is not yet adequate for general application and needs further optimization.

The objective of this study was to improve transgene expression from nuclear genome of *Chlamydomonas* by addressing some of the fundamental questions related to transgene expression in *Chlamydomonas* including identification of the best gene expression regulatory elements and optimization of the long-term, stable expression in the nucleus. Even though a large number of gene promoters have been identified for transgene expression in *Chlamydomonas*, no comprehensive study has been conducted to compare and identify the best gene promoters for transgene expression. Similarly, no effort has been made to identify optimal gene terminators, which play an important role in transgene expression by contributing to mRNA stability. In the present study, we investigated the effect of various combinations of seven different promoters and three different terminator elements on transgene expression in *Chlamydomonas*. In addition, factors affecting transformation efficiency and the stability of transgene expression were also evaluated. We also discuss results on the expression of a human recombinant protein, butyrylcholinesterase, in *Chlamydomonas*.

2. Materials and methods

2.1. Algal strains and cultural conditions

Chlamydomonas strain CC424 (*cw15*, *arg2*, *sr-u-2-60 mt-*) was obtained from the *Chlamydomonas* culture collection at Duke University, USA. Strains were grown mixotrophically in liquid or on solid TAP medium [1] at 23 °C under continuous white light (40 μE m^{-2} s^{-1}), unless otherwise stated. Medium was supplemented with 100 μg/mL of arginine when required. For the selection of transformants, solid TAP medium was supplemented with 50 μg/mL of paromomycin and 100 μg/mL arginine. For induction of *Fea1* promoter driven luciferase expression, transformants were first grown in liquid TAP medium until log phase (OD_{750} = 0.8 to 1.0). Cells were then pelleted, washed once with low iron TAP medium (Fe free TAP supplemented with 1 μM of Fe-EDTA) and resuspended in low iron TAP medium for growth for 24 h. The low iron TAP medium contains only about 6% of iron (1 μM) found in the regular TAP medium (17 μM of iron).

2.2. Vector construction

Plasmid pHsp70A/Rbcs2-cGLuc [26] carrying codon-optimized *Gaussia princeps* luciferase for *Chlamydomonas* nuclear expression was obtained from the *Chlamydomonas* culture collection at Duke University, USA. Luciferase coding sequence was amplified using primers LUCFwd1 (5′-ATCTACATATGCTCGAGATGGGCGTGAAGG-3′) and LUCRev1 (5′-AAGATAAGCTTCTAGATTACGTATCGCCGCCAGCG-3′), introducing NdeI and XbaI sites at 5′ and 3′ ends, respectively. The luciferase coding region was cloned as NdeI/XbaI fragment into the similarly digested vector pSSCR7 [27], creating plasmid pCVAC110. The paromomycin expression cassette from vector pSL18 [28] was excised as XhoI/KpnI fragment and cloned into same sites of pBlueScriptKS + to create plasmid pCVAC112. The *B2-Tubulin* promoter-luciferase-low carbon dioxide inducible protein (*CCP1*, GenBank accession number XM001692145.1) terminator cassette from pCVAC110 was then amplified using primers B2TubP Fwd(5′-ATCTAACTAGTCTGCAGCAAGCTGGCACTTTCTTGCGC-3′) and *CCP1*rev (5′-ATCTTGTCGACAAGCTGTTCCCCTTGTTCCGC-3′) to introduce PstI and SalI sites at 5′ and 3′ ends respectively and cloned into XhoI/PstI sites of pCVAC112 thereby destroying the XhoI site to create vector pCVAC113. To create additional vectors in which luciferase expression is driven by various promoters, the *B2-Tubulin* promoter (GenBank accession number KO1809.1) from pCVAC112 was excised as PstI/NdeI and replaced by the promoter sequences of *Fea1*, *psaD* (GenBank accession number AF335592.1), *Hsp70A*/*Rbcs2*P, *Chlorella* virus DNA polymerase (*CVDP*) (GenBank accession number U42580.6), *CaMV35S* and *Actin* (GenBank accession number D50838.1) creating plasmids pCVAC115, pCVAC117, pCVAC120, pCVAC123, pCVAC129 and pCVAC130 respectively. The description of these elements is presented in Table 1. The primers used to amplify the above mentioned promoters are listed in Table 2. Luciferase amplified with primers LUCFwd1 and LUCRev1 was also cloned into NdeI/XbaI sites of vector pSL18 creating plasmid pCVAC157. *B2-Tubulin*P was amplified with primers B2TubPFwd1 and B2TubPRev1 from pCVAC113 and cloned into StuI/NdeI site of pCVAC157 replacing *psaD* promoter, creating plasmid pCVAC167. To amplify and clone the 459 base pair (bp) long *B2-Tubulin* terminator (GenBank accession number XM001693945.1) from *Chlamydomonas* genome, primers B2TubTFwd1 and B2TubTRev1 were used. The 549 bp long *psaD* terminator (GenBank accession number AF335592.1) from vectors pCVAC167 and pCVAC157 was excised as XbaI/NotI fragment and replaced with *B2-Tubulin* terminator to create plasmids pCVAC168 and pCVAC169 respectively. Plasmid pCVAC164, carrying two copies of *psaD* terminator was constructed by amplifying the 1125 bp luciferase–*psaD* terminator region from plasmid pCVAC157 using primers LucFwd1 and *psaD*3′Rev and cloned into NdeI/EcoRI sites of pSL18. Refer to Table 2 for sequence of primers used for cloning *B2-Tubulin*P and *B2-Tubulin* terminator and luciferase–*psaD* terminator. The human butyrylcholinesterase gene (*huBuChE* GenBank accession number BC018141) was codon-optimized for *Chlamydomonas* nuclear expression using the Graphical Codon Usage Analyser 2.0 (www.gcua.de). The codon-optimized version was synthesized by Epoch Biolabs, Inc. (Sugar Land, TX, USA).

Table 1
Details about the promoters evaluated for transgene expression in *Chlamydomonas reinhardtii*.

Promoter name	Promoter type	Amplified from	Length amplified 5′ from the start codon	Reference
Chlamydomonas B2-Tubulin	Constitutive	pSSCR7	246 bp	Davies et al. [31]
Chlamydomonas Fea1	Inducible	pJD100	1319 bp	Allen et al. [32]
Chlamydomonas psaD	Constitutive	pSL18	814 bp	Fischer and Rochaix [23]
Hsp70A/Rbcs2fusion	Constitutive	pSL18	796 bp	Schroda et al. [24]
Chlorella virus DNA Pol	Constitutive	Genomic DNA	1000 bp	Jung et al. [34]
CaMV35S	Constitutive	pC2301	781 bp	Tang et al. [33]
Chlamydomonas Actin	Constitutive	Genomic DNA	1000 bp	This work

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