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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

Table 2
Sequences of primers used for amplifying and cloning luciferase, various promoter and terminator elements used in this study.

Primer name	Sequence
*Fea1*Fwd	5′-ATCTAACTAGTCTGCAGTACCAGGACAGAGTGCGTGTGG-3′
*Fea1*Rev	5′-GAAATCATATGCTCGAGTGGCCTGTGTAGAAGTGG-3′
Hsp70A/Rbcs2Fwd	5′-ATCTAACTAGTCTGCAGGAGCTCGCTGAGGCTTGACATG-3′
Hsp70A/Rbcs2Rev	5′-TAGAACATATGCCTGCAAATGGAAACGGCGACG-3′
*psaD*Fwd	5′-ATTCGCTGCAGCACACACCTGCCCGTCTGC-3′
*psaD*Rev	5′-AGGATCATATGGGCTTGTTGTGAGTAGCAGTGG-3′
*psaD3′*Rev	5′-AGAATGAATTCATTGCACAGTCACGCTGTCTCCC-3′
DPolFwd	5′-ATCTCTGCAGCAAAGAACGTGACTCTTGC-3′
DPolRev	5′-AGAATATTAATTCACTTGTATTTCTAAATTATGATATCATAAG-3′
*CaMV35s*Fwd	5′-ATCGATCTGCAGATGGTGGAGCACGACAC-3′
*CaMV35s*Rev	5′-ATGCTCATATGAGAGATAGATTTGTAGAGAGAGACTGG-3′
*Actin*Fwd	5′-ATCTTCTGCAGAGGTGCATGCGCTCCACGCATTAG-3′
*Actin*Rev	5′-AAGATCATATGTTTGAATCCTGCGTGTCACGTCCGC-3′
B2TubPFwd1	5′-ATCTTAGGCCTTTCTTGCGCTATGACACTTCCAGC-3′
B2TubPRev1	5′-TAATAGCATATGAGTGGCCTGTGTAGAAGTGGTAGTG-3′
B2TubTFwd1	5′-CTTATTCTAGATGCCGGCACCTCCATGCG-3′
B2TubTrev1	5′-AAGATGCGGCCGCGTGTATCTTATTACACGTGCACGAGTGTGTGG-3′

Luciferase without a stop codon was amplified with primers LUCFwd1 (5′-ATCTACATATGCTCGAGATGGGCGTGAAGG-3′) and LUCRev2 (5′-AA AGATGAATTCAAGCTTCGTATCGCCGCCAGCGCC-3′), introducing sites NdeI and EcoRI, respectively and cloned into NdeI/EcoRI sites of pSL18 creating plasmid pCVAC158. The codon-optimized *huBuChE* gene was then amplified with forward primer BF (5′-ACTTGAATTCATGCACAG CAAGGTGACCATCATCTGCATCCGCTTCCTGT-3′) and reverse primer BRHis (5′-ATCTTTCTAGATTAGTGGTGGTGGTGGTGCAGGCCCACGC AGCTCTCCTT-3′) containing a 6×His tag and cloned into EcoRI/XbaI sites of pCVAC158 fusing luciferase to N-terminus of *huBuChE* creating plasmid pBuChE.

2.3. Nuclear transformation of C. reinhardtii

C. reinhardtii nuclear transformation was performed using the glass bead method [14]. Prior to transformation, some constructs were digested with restriction enzyme(s) either to linearize the construct or to excise the two expression cassettes carrying the selection marker and gene of interest together, from the plasmid backbone. Transformants were selected on TAP medium containing 50 μg/mL paromomycin.

In order to compare the efficiency of transformation with supercoiled plasmid, linearized plasmid or excised expression cassette, 1×10^7 *Chlamydomonas* cells were transformed with either 1 μg of supercoiled plasmid pCVAC117, or 1 μg of linearized plasmid pCVAC117 or a molar equivalent of the expression cassette derived from 1 μg of plasmid pCVAC117 in each experiment.

Table 3
List of plasmids used in this study and the gene promoters and terminators flanking luciferase.

Plasmid name	Promoters and terminators flanking luciferase gene	
	Promoter	Terminator
pCVAC113	*Chlamydomonas B2-Tubulin*	*Chlamydomonas CCP1*
pCVAC115	*Chlamydomonas Fea1*	*Chlamydomonas CCP1*
pCVAC117	*Chlamydomonas psaD*	*Chlamydomonas CCP1*
pCVAC120	*Chlamydomonas Hsp70A/Rbcs2*	*Chlamydomonas CCP1*
pCVAC123	*CVDP*	*Chlamydomonas CCP1*
pCVAC129	*CaMV35S*	*Chlamydomonas CCP1*
pCVAC130	*Chlamydomonas Actin*	*Chlamydomonas CCP1*
pCVAC157	*Chlamydomonas psaD*	*Chlamydomonas psaD*
pCVAC164	*Chlamydomonas psaD*	Two copies of *Chlamydomonas psaD*
pCVAC167	*Chlamydomonas B2-Tubulin*	*Chlamydomonas psaD*
pCVAC168	*Chlamydomonas B2-Tubulin*	*Chlamydomonas B2-Tubulin*
pCVAC169	*Chlamydomonas psaD*	*Chlamydomonas B2-Tubulin*

2.4. Luciferase assays

Luciferase assays were performed at room temperature with a POLARstar OPTIMA microplate luminometer (BMG Labtech, Germany) with automated substrate injection. For assessing luciferase expression, *Chlamydomonas* transformants were inoculated into 96 well (Nunc, Rochester, NY, USA) plates containing 1 mL TAP media supplemented with arginine and grown for 2 to 3 days under continuous light. One hundred microliters of algal culture was diluted with 100 μL of TAP medium in a 96 well plate and luminescence was recorded after automatic injection of 10 μL coelenterazine (0.1 mM in 10% ethanol) (NanoLight Technologies, Pinetop, AZ, USA) every 4 s for a total of 24 s at the highest signal amplification. For calculating relative luminescent units (RLU), a standard curve of *Chlamydomonas* cell number versus OD750 was generated (Supplementary Fig. 1) and luciferase activity of each culture was normalized to the OD_{750} of the culture. Secretion of luciferase into the culture medium was confirmed by measuring luciferase activity in the culture supernatant using the following fractionation procedure. 100 μL of culture supernatant obtained following centrifugation at 12,000 *g* was mixed with 100 μL of liquid TAP medium in a 96 well plate for measuring luminescence.

2.5. Genomic DNA isolation and Southern blot analysis

Genomic DNA was isolated from 1.0×10^7 cells using a Qiagen Plant DNAeasy Kit following the manufacturer's protocol. For Southern blot, 5.0 μg of genomic DNA was used. Genomic DNA from CC424/pCVAC117 and CC424/pCVAC157 clones was digested with SacI and XhoI respectively at 37 °C for 16 h and separated on 1% (w/w) agarose gel in Tris–Borate–EDTA buffer. Following acid hydrolysis (0.25 M HCL), neutralization (0.4 M NaOH/0.6 M NaCl and 1.5 M NaCl/0.6 M Tris, pH 7.5), and equilibration in 10× sodium chloride–sodium citrate (SSC) buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0), capillary transfer with 10× SSC was set to transfer DNA samples to a positively charged nylon-66 membrane. DNA fragments were cross-linked by UV-irradiation in a Stratalinker® (Stratagene, La Jolla, CA, USA). Membranes were rinsed briefly in double distilled water and pre-hybridized with a DIG-Easy-Hyb solution (Roche, Mannheim, Germany) at 40 °C for 60 min. Heat-denaturated, DIG labeled *Gaussia* luciferase probe made using luciferase PCR product as template with the DIG-High Prime Kit (Roche) was used for overnight hybridization at 50 °C in DIG-Easy-Hyb solution. Detection was completed with the DIG Wash and Block Buffer set (Roche)

and the anti-digoxigenin (DIG)-alkaline phosphatase conjugate antibody following the manufacturer's protocol.

2.6. Real time PCR

Total cellular RNA was extracted from 1.0×10^7 cells using a Nucleospin RNAII Kit (Clonetech, Mountain View, CA, USA) according to the manufacturer's instructions. Contaminating genomic DNA was removed by treating RNA samples with DNAase I (Promega, Madison, WI, USA) following the manufacturer's instructions. RNA quality and concentration was measured using a Nanodrop (Thermo-scientific, Wilmington, DE, USA) spectrometer at 260 nm. Structural integrity of the RNAs was checked with non-denaturing agarose gel and ethidium bromide staining. 2.0 μg of total RNA was used for cDNA synthesis using the Quantas cDNA Synthesis (Quantas, USA) following the manufacturer's instructions. Real-time quantitative RT-PCR was carried out using an ABI-Step One Plus (Applied Biosystems, Foster City, CA, USA) using the PerfeCTa™ SYBR® Green FastMix™ (ROX dye) (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. The *Chlamydomonas* CβLP gene (CβLP Fwd 5′-AGTG CAAGTACACCA TTGGCGA-3′ and CβLP Rev 5′-CTTCAGCTTGCAGTTG GTCAGGTT-3′) was used as reference gene/internal control and was amplified in parallel with the target Luciferase gene (LUCRealFwd 5′-CACGCCGAAGATGAAGAAGTTCATTCC-3′ and LUCRealRev 5′-CTTCTTG AGCAGGTCGGAACACTG-3′) allowing gene expression normalization and providing quantification. Reactions were carried out with 10 ng of cDNA. All the primers were designed using the Primer Express software following the manufacturer's guidelines. For each sample, reactions were set up in quadruplicates and two biological experiments were done to ensure the reproducibility of the results. The quantification of the relative transcript levels was performed using the comparative C_T (threshold cycle) method [29]. The calculated efficiency of the CβLP and the luciferase primers as a function of template concentration was 98.59% and 103.5%, respectively (Supplementary Fig. 3).

2.7. Western blot analysis

One milliliter of cells having an $OD_{750} = 1.0$ was pelleted in a microcentrifuge at 10,000 *g* for 1 min at room temperature, resuspended in 50 μL of 1× Laemmli loading dye (0.063 M Tris–HCl pH 6.8, 2% (w/v) SDS-PAGE, 10% (v/v) glycerol, 5% B-mercaptaethanol, 0.05% (w/v) bromo-phenol-blue), and incubated at 100 °C for 5 min. Samples were removed from the water bath, cooled to room temperature, centrifuged at 10,000 *g* for 2 min, and the cell extract was separated on 10% (w/v) SDS polyacrylamide gel by electrophoresis. Protein samples were transferred to PVDF membrane (Millipore, USA) by semi-dry blotting. Membrane was incubated with anti G-LUC rabbit polyclonal antibody (Nanolight Technologies, USA) at 1:1000 dilution at 4 °C overnight following blocking the membrane with 0.5% (w/v) BSA in TBS containing 0.05% (v/v) Tween-20. Anti-rabbit IgG antibody (Sigma, USA) conjugated to horse-radish peroxidase was used as secondary antibody at 1:10,000 dilution and detection was completed by chemiluminescence using luminal (0.4 mM) and iodophenol (8.0 mM) (Sigma, Saint Louis, MO, USA) [30]. Quantitation of protein expression was done using luciferase standards (NanoLight Technologies, Pinetop, AZ, USA) with ImageJ software.

3. Results and discussion

3.1. The Chlamydomonas psaD promoter is an excellent promoter for nuclear transgene expression in C. reinhardtii

Seven promoters were evaluated to identify the best promoter for driving transgene expression in *Chlamydomonas* (Table 3). Among these seven promoters, five promoters were *Chlamydomonas* promoters, including *B2-Tubulin* [31], *Fea1* [32], *psaD* [23], *Actin* and the *Hsp70A/Rbcs2* fusion promoter [24] (Table 1). Two heterologous viral promoters were also evaluated for transgene expression in *Chlamydomonas*. Since no viruses infecting *Chlamydomonas* are known, we decided to test the *Chlorella* virus DNA polymerase promoter (*CVDP*) promoter obtained from a virus that infects *Chlorella* NC64A (*Paramecium bursaria Chlorella* virus-1 (PBCV-1)). The *CVDP* promoter was selected because it has been demonstrated to perform as well as the Cauliflower Mosaic virus 35S (*CaMV35S*) promoter [33] for driving transgene expression in *Chlorella* [34]. The most preferred promoter for transgene expression in plants, the Cauliflower Mosaic virus 35S (*CaMV35S*) promoter was also tested. With the exception of the *Fea1* promoter, which is induced under iron deficiency, all other *Chlamydomonas* promoters tested in this study are known to have some constituent expression under standard growth conditions, hence the uniform expression of the *Chlamydomonas* promoters being tested is assumed, but not documented (see references cited in Table 1). Importantly, the *Hsp70A/Rbcs2* promoter harbors an intron in the 5′UTR region that is known to enhance transgene expression [35]. Similar to *Hsp70A/Rbcs2* promoter, the *Actin* promoter also contains an intron in the 5′UTR. Except for the *Fea1*, *Actin* and *CVDP* promoters all other promoters have been previously used for transgene expression in *Chlamydomonas*. Additional information about the size and source of the promoters used in this study is presented in Table 1.

To determine the levels of transgene expression, luciferase (obtained from *G. princeps*) activity, driven by the promoter of interest, was assayed as the reporter. A codon-optimized *Gaussia* luciferase gene for *Chlamydomonas* nuclear expression [26] was obtained from the *Chlamydomonas* culture collection at Duke University, USA. All the luciferase expression constructs were identical in all aspects including the terminator (*CCP1*), the paramomycin resistance cassette, and the vector backbone. The constructs differed only for the promoter driving luciferase expression (Fig. 1). Each construct was restriction digested either to linearize the plasmid or to excise the expression cassettes carrying the selection marker and gene of interest from the plasmid backbone before *Chlamydomonas* transformation. For each construct, 83 transformants selected on agar plates carrying paromomycin were screened for luciferase activity. Since the integration of transgene into the nuclear genome of *Chlamydomonas* occurs via nonhomologous recombination, the transformants exhibited a broad range of luciferase activity. Nine clones showing the highest luciferase activity for each construct were selected for carrying out relative luminescent unit (RLU) measurements. Induction of *Fea1* promoter driven luciferase expression was performed by washing and subsequent growth of log phase cultures ($OD_{750} = 0.8–1.0$) in low iron TAP medium (Fe free TAP supplemented with 1 μM of Fe-EDTA) for 24 h. Luciferase activity was normalized to the OD_{750} of the culture to calculate RLU. The data was analyzed following single factor analysis of variance (ANOVA).

As shown in Fig. 2, significant differences in luciferase expression levels were observed among clones expressing luciferase under the control of different promoters. The lowest luciferase expression was observed in transformants in which luciferase expression was driven by the *Fea1* promoter. Luciferase expression in all other transformants was normalized to the levels of expression observed in the *Fea1* transgenics. The highest levels of luciferase expression were observed in luciferase transformants having the *psaD* promoter (85×), followed by the *Actin* (61×), *Hsp70A/Rbcs2* (40×) and *B2-Tubulin* (18×) promoters (Fig. 2A). Among *Chlamydomonas* constitutive promoters, the lowest expression levels were observed with *B2-Tubulin* promoter (18×). Results of the RLU experiments were confirmed by western blot analysis of the luciferase protein levels in transgenic *Chlamydomonas* clones. A strong positive correlation was observed between RLU and luciferase protein levels as evidenced by the results of the western blot (Fig. 2B). The highest luciferase protein levels were observed in clones expressing luciferase under the control of *psaD* promoter, which also exhibited the highest RLU (Fig. 2A and B). Luciferase protein expression levels in the

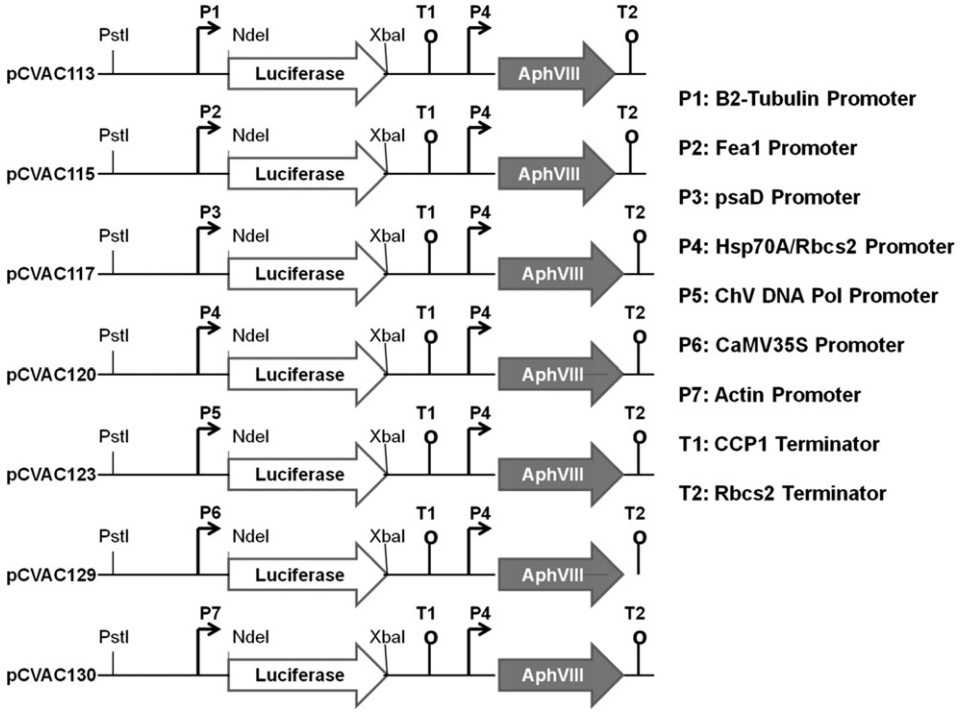

Fig. 1. Plasmid constructs used to compare seven gene promoters for transgene expression in *Chlamydomonas reinhardtii*. All plasmid constructs in this figure were identical in all aspects but one; they differed with respect to the promoter driving luciferase expression. All the promoters were cloned into the PstI/NdeI sites of vector pCVAC113. All vectors carried the *AphVIII* gene as a selectable marker that confers resistance to paromomycin and is driven by *Hsp70A/Rbcs2* hybrid promoter.

psaD promoter clones were followed in descending order by *Actin*, *Hsp70A/Rbcs2*, *B2-Tubulin* and the *CaMV35S* promoter clones based on western blot analysis. These results were consistent with the respective RLUs (Fig. 2A).

Surprisingly the heterologous viral promoters *CVDP* (2× RLU) and *CaMV35SP* (5× RLU) performed poorly relative to constitutive endogenous promoters. Several heterologous viral promoters, including *CaMV35S* are widely used for transgene expression in plants.

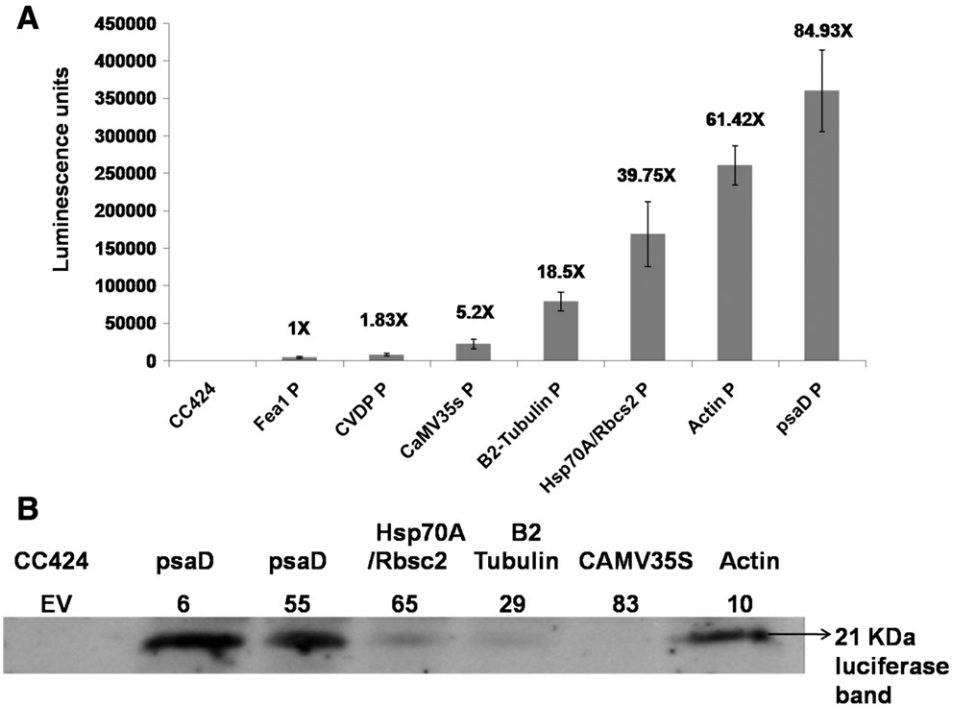

Fig. 2. Relative expression levels of various gene promoter:terminator constructs. A) Comparison of Relative Luminescence Unit (RLU) activities in different transgenics. Nine clones showing the highest luciferase activity from each construct were selected for carrying out RLU measurement. RLU was calculated by normalizing the luciferase activity exhibited by each culture to the optical density (OD_{750}) of the culture. The data was analyzed by single factor ANOVA. The calculated F value was significantly higher than the table F value at $P = 0.05$ indicating that there were significant differences among the clones expressing luciferase under the control of different promoters. B) Western blot analysis of luciferase levels in *Chlamydomonas* strains transformed with different plasmid constructs (pCVAC117, pCVAC120, pCVAC113, pCVAC129 and pCVAC130). EV stands for empty vector. The numbers below the plasmids refer to individual transformant numbers. Colonies showing highest luciferase expression were selected from each construct for western analysis. Cells equivalent to $OD_{750} = 1$ were loaded from each culture. Blot was probed with anti-*Gaussia* luciferase antibody.

However, there are significant differences between the AT–GC composition of the host organisms infected by the CaMV and PCBV and *Chlamydomonas* which could have significant impact on the ability of these viral promoters to drive gene expression in *Chlamydomonas*. The GC-content of *Chlamydomonas* genome is high, about 63% [4], while that of the *CaMV35S* and *CVDP* promoters is low, 46% and 42%, respectively. Therefore, the *Chlamydomonas* transcriptional machinery may not efficiently initiate transcription from the viral promoters leading to poor expression of transgenes driven by these promoters.

The *psaD* gene codes for the alpha subunit of photosystem I. The coding sequence of *psaD* lacks any introns which may account for the ability of *psaD* promoter to drive strong expression of transgenes lacking introns [23]. To determine whether the *psaD* promoter has any unique transcription factor binding sites that are not present among the other *Chlamydomonas* promoters tested in this study, the nucleotide sequences of *Chlamydomonas B2-Tubulin*, *psaD*, *Actin* and *Hsp70A*/*Rbcs2* fusion promoters were subjected to analysis by the Plant cis-Acting regulatory DNA Element (PLACE) software (http://www.dna.affrc.go.jp/PLACE/signalscan.html) [36]. Significantly, we failed to observe any such unique trans-acting factor domains in the *psaD* promoter sequence.

3.2. The psaD terminator significantly improves nuclear transgene expression in Chlamydomonas when paired with other gene promoters

Most research focusing on optimizing transgene expression in plants and *Chlamydomonas* has focused on identification of strong gene promoters to maximize transgene expression, while little effort has been made to identify optimal transcription terminators so as to improve transgene expression. A transcription terminator is a sequence downstream from the 3′ end of an open reading frame that signals the RNA polymerase to release the newly made RNA molecule. Except for few studies in plants that compared various terminators [37–39], no published report on the effect of gene terminators on transgene expression in *Chlamydomonas* is available.

Hence, we decided to test the impact of different gene terminators on luciferase expression driven by two different *Chlamydomonas* gene promoters. Terminator regions of the *CCP1* [40,41], *B2-Tubulin* and the *psaD* genes were selected for this study. Most of the well characterized plant and *Chlamydomonas* terminator regions have a poly-A site located within a region of 200 to 300 nucleotides downstream of the stop codon. A 550 bp region of *CCP1*, 459 bp region of *B2-Tubulin* and 549 bp region of the *psaD* terminators consisting of sequences starting immediately downstream of the stop codon and including the polyadenylation site were tested for this study. Six constructs were made using the three selected terminator regions in which the luciferase expression was driven by *B2-Tubulin* and *psaD* promoters (Fig. 3). A construct with two copies of *psaD* terminator was also tested since the use of double terminators has been reported to enhance transgene expression [42,43]. *Chlamydomonas* strain CC424 was transformed with these constructs. Ninety three transformants selected on TAP–paromomycin plates were screened for luciferase activity from each construct. Nine clones exhibiting the highest luciferase activity were selected from each transformation screen to carry out the RLU assay. The data was analyzed using a single factor ANOVA. The lowest luciferase expression was observed with the *B2-TubulinP–LUC–CCP1* terminator construct, which was used as a baseline (1×) to calculate relative luciferase expression for the other constructs. Significantly, highest luciferase expression levels were observed in clones expressing luciferase with the *psaD* terminator, irrespective of the promoter driving luciferase expression. Overall, the *psaDP–LUC–psaD* terminator construct had the highest (48×), relative luciferase expression levels followed by *B2-TubulinP–LUC–psaD* terminator (39×), the *psaDP–LUC–double psaD* terminator (17×), the *psaDP–LUC–CCP1* terminator (9×), the *psaDP–LUC–B2-Tubulin* terminator (8×), and the

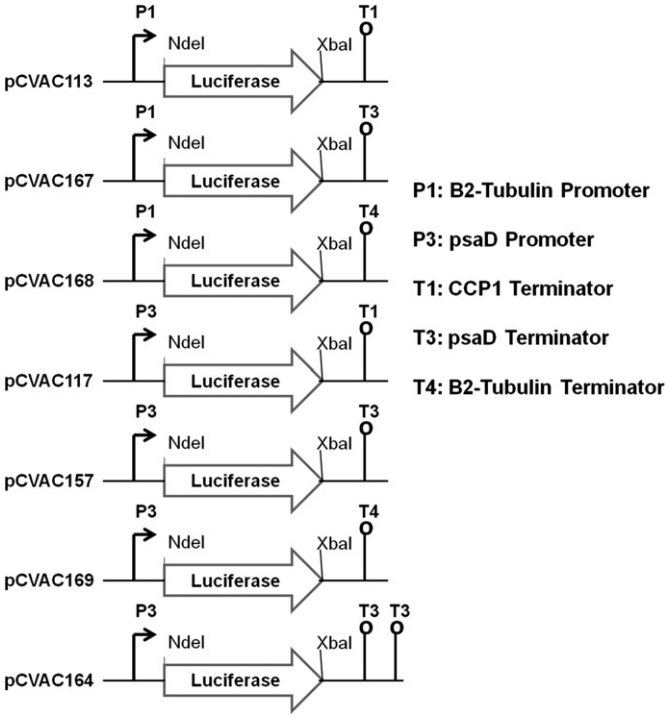

Fig. 3. Schematic drawings of all plasmid constructs used to study the effect of three different terminator regions on luciferase expression. All the vectors carry the *AphVIII* gene as selectable marker that confers resistance to paromomycin and is driven by *Hsp70A*/*Rbcs2* fusion promoter.

B2-Tubulin–LUC–B2-Tubulin terminator (4×) construct (Fig. 4). Consistent with these observations, transgenics in which luciferase expression was driven by the *psaD* promoter but coupled with either the *CCP1* or the *B2-Tubulin* terminator had lower luciferase expression levels than when luciferase expression was driven by the *psaD* promoter coupled with the *psaD* terminator. Interestingly, inclusion of an additional copy of the *psaD* terminator did not improve luciferase expression; in fact it reduced luciferase expression by 63% when compared to the single copy *psaD* terminator construct, pCVAC157 (Fig. 4).

The possible means by which the *psaD* terminator could enhance transgene expression include more efficient transcript processing, enhancing transcription (cis effect), or by enhancing transcript stability. To test whether the high RLU observed with *psaDP–LUC–psaD* terminator clones was due to enhanced transcription or enhanced stability of the transcript conferred by the *psaD* terminator, three single copy transformants of pCVAC117 (*psaDP–LUC–CCP1* ter) and pCVAC157 (*psaDP–LUC–psaD* ter) were identified by Southern blot (Supplementary Fig. 2A and B) and real time PCR was used to quantify the luciferase transcript levels among these single copy transformants. Luciferase transcript levels in the pCVAC157 clones were between 1.5- and 25-fold higher than luciferase transcript levels in the pCVAC117 clones (Fig. 5A). The highest luciferase transcript levels were observed in CC424/pCVAC157 clone 84. Similar results were observed in other CC424/pCVAC157 clones. These results indicate that higher luciferase expression among clones carrying *psaD* terminator is associated with an increase in the steady state levels of luciferase transcripts. The strong positive correlation ($r^2 = 0.7499$) (Fig. 5E) observed between luciferase enzyme activity levels and steady-state luciferase transcript levels indicates that the increase in steady-state luciferase transcript levels was primarily responsible for the high luciferase enzyme activity observed among CC424/pCVAC157 clones. The level of luciferase expression among single copy clones of pCVAC117 (clones 18 & 25) and pCVAC157

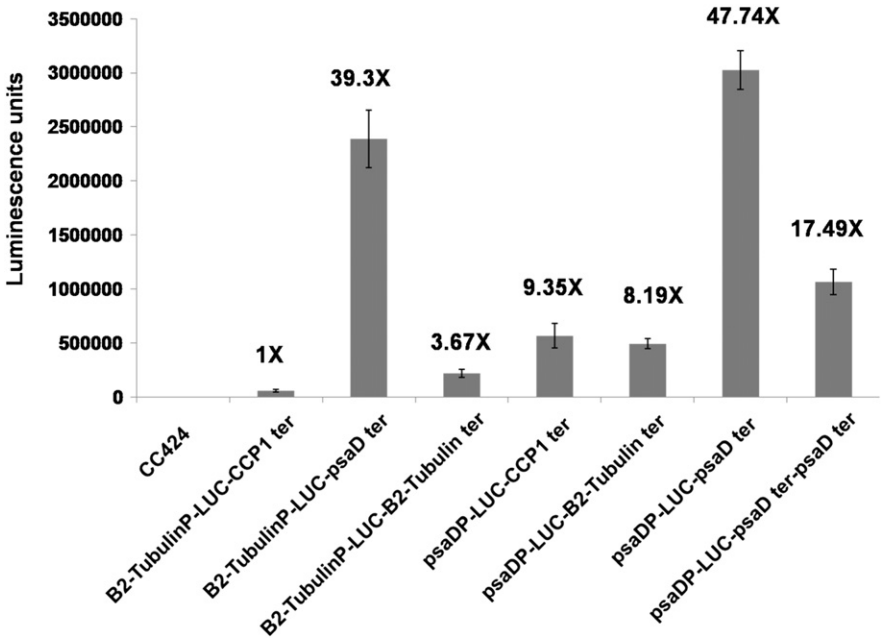

Fig. 4. Comparison of relative luciferase (RLU) activity in plasmids with different gene terminators. Nine clones showing highest luciferase activity from each construct were selected for carrying out RLU measurements. RLU was calculated by normalizing the luciferase activity exhibited by each culture to the OD_{750} of the culture. The data was analyzed by single factor ANOVA. The calculated F value was significantly higher than the table F value at $P = 0.05$ indicating that there were significant differences among the clones expressing luciferase with different terminator regions. $N = 72$.

(clones 48 & 84) was then checked by western blot analysis (Fig. 5B). The results of western blot support the observations of the RLU and real time PCR experiments. The highest immuno-detectable levels of luciferase were observed in the CC424/pCVAC157 clones that exhibited highest luciferase enzyme and transcript levels. The level of luciferase expression among single copy clones of pCVAC117 and pCVAC157 was also quantified by western blot using luciferase standards as control. The amount of luciferase accumulated by cells representing 1 mL of $OD_{750} = 1.0$ culture was estimated to be about 10 ng for pCVAC157 clone 84 (Fig. 5C). The supernatant fraction of the same culture was estimated to contain about 20 ng of luciferase (Fig. 5D). The total luciferase accumulation was equivalent to 0.4% of the total soluble protein in the algal cell.

Since the terminator regions are implicated in regulating gene expression it's possible that the *psaD* terminator is more efficient at transcriptional termination and/or 3′ end processing than the *B2-Tubulin* or *CCP1* terminators. Previous studies in plants have demonstrated that the choice of gene terminator may substantially impact gene expression levels. Ingelbrecht et al. [38] observed a 60-fold higher nptII expression in transgenic tobacco plants expressing the npt-II gene under the control of the *CaMV35S* promoter coupled with the *RBCS2* terminator relative to identical constructs using the chalcone synthase terminator. In addition, Nagaya et al. [39] observed a two fold increase in GUS expression in *Arabidopsis* plants using the *Hsp18.2* terminator relative to the *Nos* terminator. In addition, a double *CaMV35S*/*Nos* terminator as well as heterologous terminator pairs including the *CaMV35S* and *Nos* terminators has been reported to enhance transgene expression by 3- to 4-fold compared to single copies of these terminators [42,43].

3.3. Impact of plasmid structure on transformation efficiency and transgene expression

Previously, Kindle et al. [14] reported that the structure of transforming DNA element (plasmid versus linear DNA) had an impact on transformation efficiency. They demonstrated that the use of linear DNA for transformation increased the probability of its integration into the host genome presumably due to the presence of free DNA ends. To determine whether supercoiled, linearized plasmid, or excised expression cassette gave the highest transformation and gene expression frequency, we transformed *Chlamydomonas* with equivalent molar concentrations of supercoiled, linearized, and double digested (excised expression cassette) pCVAC117 plasmid DNA. Equal numbers of *Chlamydomonas* cells were transformed with 1 μg of supercoiled plasmid, 1 μg of plasmid linearized with KpnI or PstI, or 1 μg of plasmid double digested with KpnI and PstI to generate a linear expression cassette and a separate plasmid vector backbone sequence. The expression cassette obtained with KpnI/PstI double digestion was used along with the vector backbone for *Chlamydomonas* transformation. Transformation efficiency was measured by counting all the paromomycin resistant colonies from each of three independent experiments. The transgenics were then assayed for luciferase expression to calculate the percentage of colonies exhibiting luciferase expression. All the data was analyzed by single factor ANOVA. The highest number of paromomycin resistant transformants was recovered with plasmid DNA in which the expression cassette had been excised (207 transformants) from the vector backbone, followed by Kpn1 linearized plasmid (202 transformants), Pst1 linearized plasmid (148 transformants), and supercoiled plasmid (88 transformants) (Fig. 6A). With respect to the number of paromomycin resistant transformants recovered, no significant difference was found between Kpn1 linearized plasmid (202 transformants) and expression cassette treatments (207 transformants) (Fig. 6A). However, the number of paromomycin resistant transformants recovered with KpnI linearized plasmid (202 transformants) and expression cassette treatments (207 transformants) were significantly higher than the number of transformants recovered with Pst1 linearized (148 transformants) and supercoiled plasmid (88 transformants) treatments (Fig. 6A). The Pst1 linearized treatment also produced significantly higher number of transformants when compared to the supercoiled plasmid treatment. Overall, the highest number of transformants (207 transformants) was observed when the expression cassette was excised

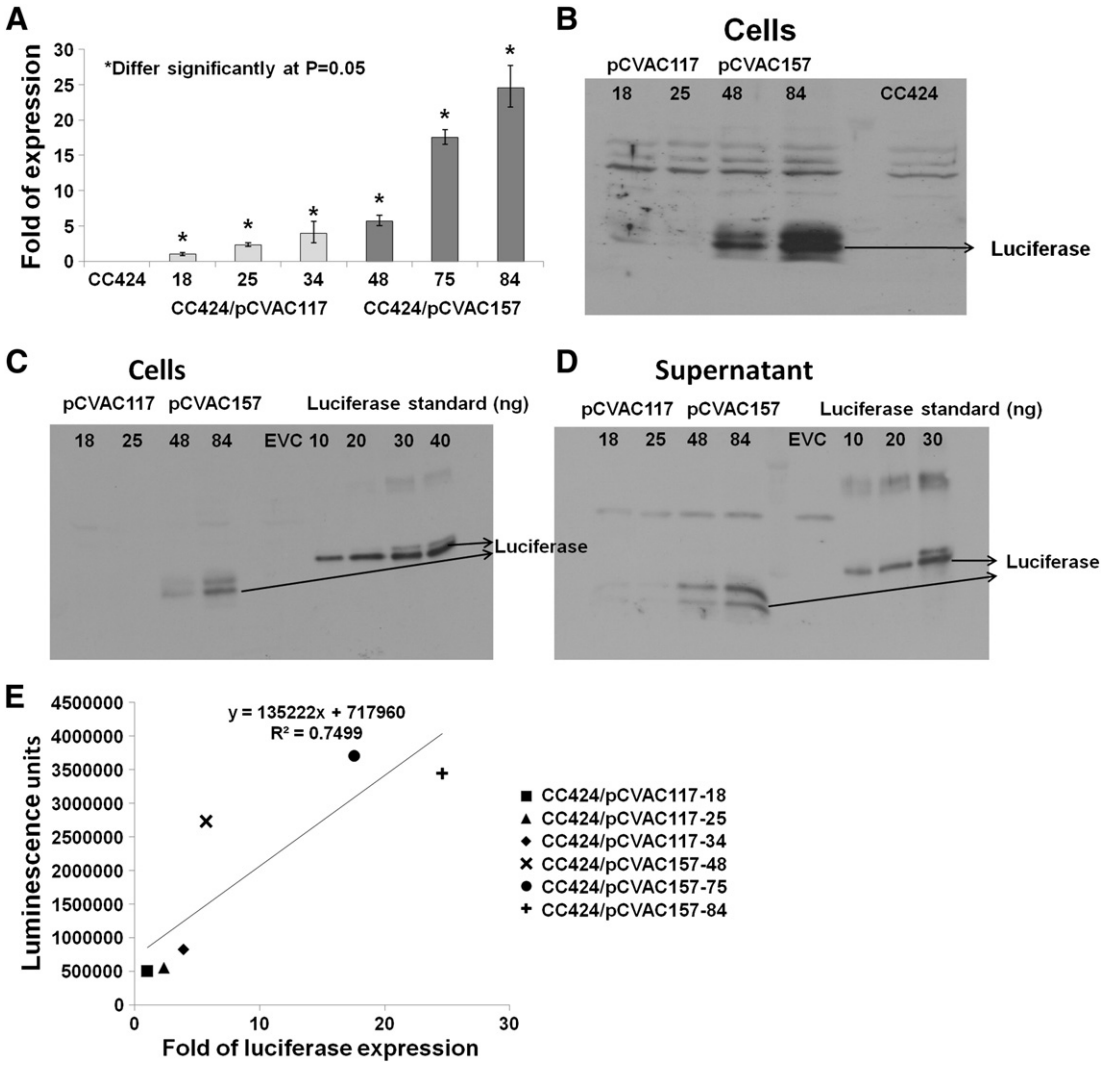

Fig. 5. Real-time PCR and western blot analysis of selected single transgene integration events from pCVAC117 and pCVAC157 constructs. A) Estimation of luciferase transcript levels among selected single transgene integration events from pCVAC117 and pCVAC157 constructs by real-time PCR. *Chlamydomonas* CβLP gene was used for normalization. The mean ΔΔCT values from three experiments were subjected to analysis through single factor ANOVA. The calculated F value was significantly higher than the F value for P = 0.05 indicating that pCVAC117 and pCVAC157 clones differed significantly with respect to luciferase transcript levels. The experiment was repeated three times (N = 3) with four replications each time. B) Western blot analysis showing luciferase expression among selected single-copy events of pCVAC117 and pCVAC157 clones. Cells equivalent to 1 mL of culture having an OD_{750} = 1 were loaded. C) Estimation of the amount of luciferase protein accumulated by clones using luciferase standards as reference. Cells equivalent to 1 mL of culture having an OD_{750} = 1 were loaded from each culture. D) Western blot analysis of the amount of luciferase protein present in the culture supernatant of selected single copy events of pCVAC117 and pCVAC157 clones using luciferase standards as reference. Culture supernatant from 1 mL of cells having an OD_{750} = 1 culture were loaded for each sample. Blot was probed with anti-*Gaussia* luciferase antibody. E) Correlation between steady-state luciferase transcript levels as measured by real-time PCR (Fig. 5A) and luciferase luminescence units (Fig. 4) among single copy transformants of CC424/pCVAC117 and CC424/pCVAC157.

from plasmid backbone and the lowest number (88 transformants) was observed with the supercoiled plasmid (Fig. 6A). The transformation efficiency with expression cassette plus vector backbone was 2.0×10^{-5}, which was 2.35-fold higher than the transformation efficiency of 8.8×10^{-6} observed with the supercoiled plasmid.

All the paromomycin resistant transformants obtained from the above experiment were then screened for luciferase expression. While 71% of the transformants obtained with linearized expression cassette exhibited luciferase expression, the percentage for KpnI linearized plasmid transformants was 53%. For PstI linearized plasmid the luciferase expression frequency of paromomycin resistant colonies was 43% and for the supercoiled plasmid transformants it was 32% (Fig. 6B). The results indicate that transformation of *Chlamydomonas* with isolated expression cassettes is more efficient in terms of the number of transformants recovered and the percentage of transformants exhibiting expression of both transgenes (paromomycin resistance and luciferase) when compared to either linearized plasmid or supercoiled plasmid.

Currently, the mechanism by which linearized DNA enhances transformation efficiency and expression of multiple insert elements is not known, however, it is likely that linearized DNA integrates into genomic DNA more efficiently compared to the supercoiled plasmid. Non-homologous end-joining (NHEJ) appears to be the major pathway for double strand break (DSB) repairs in higher eukaryotes [44,45] and it is postulated that transgenes, including those carried on T-DNA of *Agrobacterium tumefaciens* integrate into the genomes of higher eukaryotes predominantly through NHEJ [46,47]. Since transgenes appear to integrate into the genomes of eukaryotic species via NHEJ during DSB repairs, the availability of free genomic ends/DSBs can have significant impact on transformation efficiency. Kohler et al. [48] demonstrated that integration of foreign DNA into genomes of higher plants might be limited by availability of free

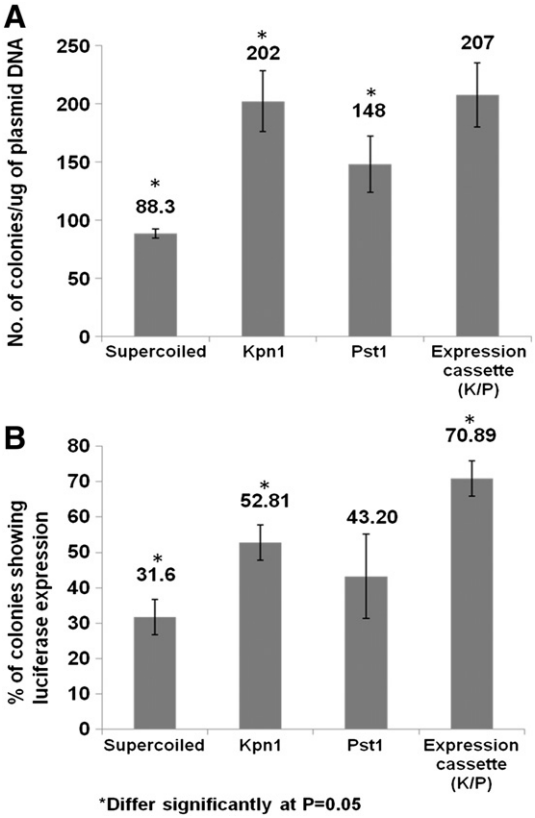

Fig. 6. Transformation and expression efficiency of algae transformed with various plasmid constructs. A) Number of paromomycin resistant colonies obtained by transforming 1×10^7 *Chlamydomonas* cells/μg of supercoiled, linearized and KpnI/PstI digested pCVAC117 (*psaDP–LUC–CCP1* ter) vector. The experiment was conducted three times. The data was analyzed by single factor ANOVA. While the F value is 4.07, the calculated F value was 9.18 at 5×10^{-3} level of significance indicating that there were significant differences among the number of transformants obtained following transformation of equal number of *Chlamydomonas* cells with either supercoiled, linearized plasmid or expression cassette alone. The calculated critical difference (CD) value is 50.53. While no significant difference was found between Kpn1 linearized plasmid and expression cassette treatments, these two treatments differed significantly from Pst1 linearized plasmid and supercoiled plasmid treatments with respect to the number of paromomycin resistant colonies recovered following *Chlamydomonas* transformation. The Pst1 linearized plasmid and supercoiled plasmid treatments also differed significantly. N = 3. B) Percentage of paromomycin resistant colonies obtained with supercoiled, linearized and KpnI/PstI digested pCVAC117 (*psaDP–LUC–CCP1* ter) vector exhibiting luciferase expression. The experiment was repeated three times. The data was analyzed by single factor ANOVA. While the table F value is 4.07, the calculated F value was 7.62 at 1×10^{-3} level of significance indicating that there are significant differences among the percentage of transformants exhibiting luciferase expression following transformation of equal number of *Chlamydomonas* cells with either supercoiled, linearized plasmid or expression cassette alone. The calculated critical difference (CD) value is 16.55. While the difference between expression cassette and other treatments was significant, no significant difference was observed among either the linearized plasmid treatments or supercoiled and Pst1 treatments. The Kpn1 treatment differed significantly from the supercoiled plasmid. N = 3.

genomic ends/DSBs. Integration of transgenes in the genomes of plant cells can be enhanced by treatment of plant cells with X-rays that induce DSBs in the genome enhancing transgene integration [48]. Similar to the availability of free genomic ends that are known to influence transformation efficiency, the availability of free ends in the transforming DNA can also enhance transformation efficiency. Thus, it is possible that linearized plasmid with free ends is more readily incorporated through NHEJ during DSB repair of *C. reinhardtii* genome compared to supercoiled plasmid and results in improved transformation efficiency.

The *Chlamydomonas* genome is GC rich (63%), while the plasmids being derived from bacteria are AT rich. Transformation of *Chlamydomonas* with linearized/supercoiled plasmid leads to integration of plasmid backbone into the *Chlamydomonas* genome. Several studies in both plant and animal systems have demonstrated that the introduction of plasmid backbone sequences along with transgene can adversely affect transgene expression [49]. The effects range from transgene silencing to transgene rearrangement and illegitimate recombination. The AT rich vector backbone sequences are also known to be recombionogenic and may promote illegitimate recombination [50]. Similarly, perhaps transformation of *Chlamydomonas* with expression cassettes does not suffer from the draw backs associated with the introduction of plasmid backbone and thus results in a significantly higher percentage of transformants expressing multiple genes of interest.

3.4. Expression of nuclear transgenes is stable in Chlamydomonas

There have been conflicting reports regarding nuclear transgene expression stability in *Chlamydomonas*. While Cerruti et al. [19] observed that about 50% of spectinomycin resistant colonies reverted to spectinomycin sensitive phenotype when grown under non-selective conditions after a few generations, stable expression of *AphVII* (hygromycin resistance) was observed by Berthold et al. [51] even after seven months of cultivation on nonselective media. Most studies evaluating transgene stability in *Chlamydomonas* have employed antibiotic selection markers. A gene of interest is often introduced into *Chlamydomonas* along with a gene encoding a selectable marker and the transformants are selected based on the expression of the selectable marker. This approach does not guarantee, however, that the co-integrating transgene of interest is also expressed. Moreover, since *Chlamydomonas* posses the machinery for gene silencing, there is a very high probability that even though *Chlamydomonas* transformants continue to grow on selection media, the expression of the gene of interest may be reduced through one of the several gene silencing mechanisms. To determine the relative expression stability of various constructs, we evaluated luciferase

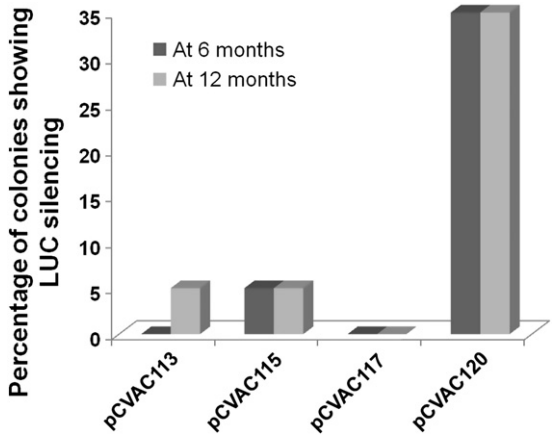

Fig. 7. Long-term stability of luciferase expression in *Chlamydomonas*. Expressed as the percentage of transgenic *Chlamydomonas* colonies from constructs pCVAC113, pCVAC115, pCVAC117 and pCVAC120 exhibiting luciferase silencing 6 months and 12 months after transformation. N = 20.

expression in clones maintained on paromomycin plates for extended periods of time.

We compared the stability of luciferase expression among 80 independent *Chlamydomonas* transformants generated using four different constructs over a period of 12 months. Twenty clones expressing luciferase activity were picked from each of the following constructs: pCVAC113, pCVAC115, pCVAC117 and pCVAC120. The pCVAC113, pCVAC117 and pCVAC120 clones were obtained using excised expression cassettes. In contrast, the pCVAC115 clones were obtained using linearized plasmid. These clones were then screened for luciferase activity 6 months and 12 months after the initial phenotypic characterization. While none of the colonies from the pCVAC113 and pCVAC117 constructs exhibited any loss of luciferase expression over 6 months, one transformant (5%) generated using the pCVAC115 construct and seven transformants (35%) generated using the pCVAC120 construct exhibited complete loss of luciferase expression after 6 months (Fig. 7). At 12 months, one transformant (5%) generated using the pCVAC113 and the pCVAC115 plasmid and seven transformants (35%) generated using the pCVAC120 construct exhibited complete loss of luciferase expression. No transformants generated using the pCVAC117 construct exhibited loss of luciferase expression after 12 months.

The results indicate that transgene silencing in *Chlamydomonas* appears to be initiated relatively early and that clones that are not affected by transgene silencing shortly after production maintain stable levels of transgene expression. The significantly high frequency of clones showing luciferase silencing for transgenics generated using the pCVAC120 construct is most likely due to the fact that in this construct the expression of the luciferase gene as well as the selection marker (*AphVIII*) is driven by the same promoter, *Hsp70A/Rbcs2* which probably leads to homology dependent transcriptional gene silencing [52,53]. Studies with plants [53,54] have also shown that the presence of more than one copy of a single promoter driving transgene expression can lead to homology-dependent transcriptional gene silencing. Homology of as little as a 90 bp region among two promoters has been shown to lead to homology-dependent gene silencing [53]. Consequently, it is recommended that expression of the selectable marker and the gene-of-interest should be driven by different promoters. Overall, the results indicate that stability of transgene expression in *Chlamydomonas* does not appear to be a major concern provided that the gene-of-interest and the selection marker are driven by different promoters.

3.5. Expression of luciferase-human butyrylcholinesterase fusion protein in *Chlamydomonas*

To validate further the *psaD–psaD* promoter–terminator expression cassette as an expression system for foreign proteins, we transformed *Chlamydomonas* with a plasmid containing the gene encoding a luciferase — human butyrylcholinesterase (huBuChE) protein fusion protein. The huBuChE is a heavily glycosylated homotetramer of 340 kDa with nine N-glycosylation sites per catalytic subunit representing almost 25% of its mass [55]. The huBuChE is expressed primarily in the liver and it is part of the acetylcholinesterase (AChE) family. It is a non-specific enzyme that hydrolyzes many

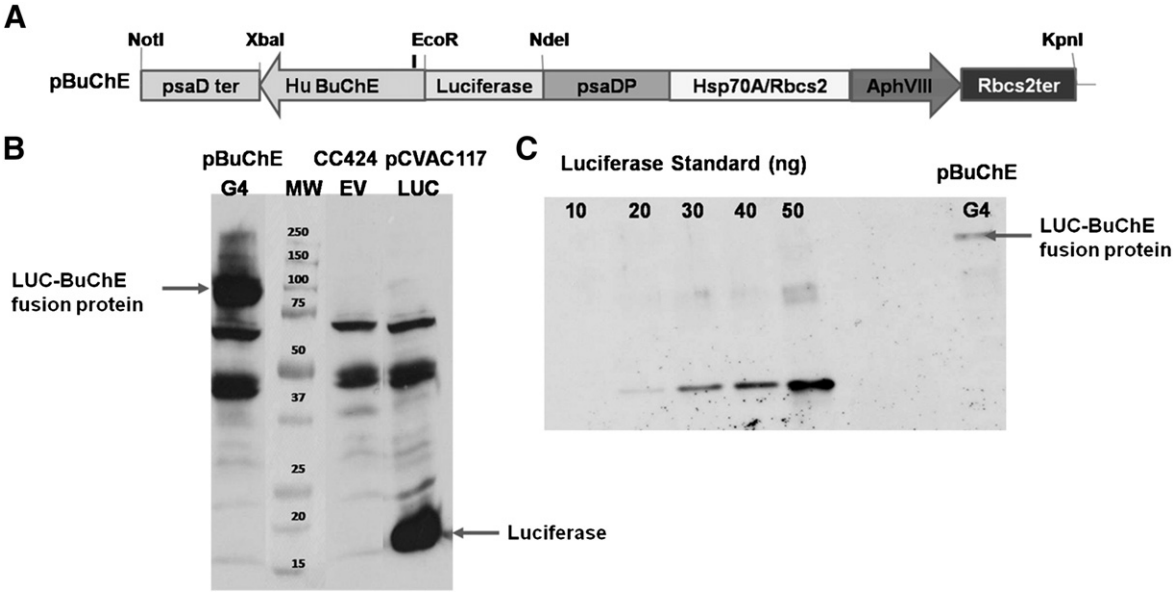

Fig. 8. Butyrylcholinesterase expression in *Chlamydomonas*. A) Schematic drawing of pBuChE construct, B) Western blot analysis of *Chlamydomonas* strain transformed with pBuChE. The colony showing highest luciferase expression (pBuChE G4) was selected for western blot analysis. Wild-type cells transformed with empty vector (CC424 EV) and with luciferase expression vector pCVAC117 (pCVAC LUC) were used as negative and positive controls, respectively. Cells equivalent to 1 mL of OD750 = 1 were loaded from each culture. MW: Protein standard. C) Western blot using luciferase standards to determine level of expression of LUC-BuChE fusion protein in *Chlamydomonas* clone pBuChE G4, 7 μg of total soluble protein was loaded from clone pBuChE G4 and probed with anti *Gaussia* luciferase antibody.

different choline esters, however, its exact physiological function is still unknown. The huBuChE can also effectively scavenge organophosphates providing protection against organophosphate nerve gas agents [56] used in warfare and as insecticides. Nerve gases are highly toxic organophosphates or esters of phosphoric acid [57]. These neurotoxins disrupt the mechanism by which nerves transfer messages to organs. According to the World Health Organization approximately 3 million cases of pesticide poisonings occur every year worldwide resulting in over 200,000 deaths [58].

To facilitate screening for the expression of foreign proteins, we fused luciferase to the N-terminus of huBuChE. Attempts to express the huBuChE alone gave very poor expression results. Since luciferase was well expressed in *Chlamydomonas*, we fused it to the N-terminus of huBuChE to see if we could get high levels of expression of the fusion protein. The *LUC–huBuChE* fusion was cloned into the *psaDP–psaD* promoter–terminator expression cassette of vector pSL18 (Fig. 8A) and transformation was performed with the expression cassette via the glass bead method to reduce the copy number of integrated transgenes. The transformation generated thousands of colonies, from which 300 colonies were screened for luciferase activity. The transformant exhibiting the highest luciferase activity was tested for expression of *LUC*–huBuChE fusion protein through immunoblot analysis using anti-G-*LUC* antibody (Fig. 8B). A protein band of approximately 90 kDa corresponding to the predicted size of the *LUC*–huBuChE fusion protein was observed in the transformant. In addition to the 90 kDa band, two other bands of approximately 160 kDa and 270 kDa were also observed in the transformant, which may represent glycosylated or oligomerized states of the protein, since in its natural state huBuChE is a glycosylated tetramer [55]. Furthermore, it has been shown that the amphiphilic character of huBuChE leads to oligomerization [59]. The level of expression of *LUC*–huBuChE fusion protein was estimated by densitometry of the immunoblot using known amounts of luciferase standards in the linear range of detection sensitivity (Fig. 8C). Based on the intensity of the bands the estimated level of *LUC*–huBuChE fusion protein was equivalent to 0.4% of total soluble protein in the algal cell. This expression level (0.4% of total soluble protein) is comparable to the expression levels of huBuChE obtained in plants [60]. These results demonstrate that proteins that may be difficult to express by themselves may be expressed at high levels when fused to a foreign protein (luciferase) that is well expressed in *Chlamydomonas*. However, the *LUC*–huBuChE fusion protein was enzymatically inactive, a common problem with expression of many fusion proteins that must be addressed on a case-by-case basis [60,61].

In conclusion, the present work demonstrates that transgene expression from *C. reinhardtii* can be significantly enhanced by using appropriate expression cassette. The results of this study also revealed that it is not only the gene promoter elements that influence transgene expression, but that gene terminator elements can also significantly impact expression levels of a transgene. The findings of this work also demonstrated that the transformation and transgene expression efficiency in *C. reinhardtii* can be significantly improved by using linearized transgene expression cassettes for transformation. The results of this study in conjunction with the advantages that *Chlamydomonas* offers over plants in terms of faster multiplication rates (8 h), the ability to obtain stable transgenic lines in a period of 3–4 weeks, and the ability to scale-up biocontained production within a short period of time make *C. reinhardtii* an organism worth considering for production of recombinant proteins. Moreover, unlike crop plants whose cultivation and harvest yield is dictated by seasons and weather, *C. reinhardtii* can be grown in continuous production cycles in a closed bioreactor which also provides strict control over the escape of transgenic material. The abovementioned advantages of *C. reinhardtii*, along with the findings of this study, make a strong case in favor of using *C. reinhardtii* as a biofactory for the production of foreign proteins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2013.09.002.

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