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Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*

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ABSTRACT

Microalgae are diverse photosynthetic microbes which offer the potential for production of a number of high value products (HVP) such as pigments, oils, and bio-active compounds. Fast growth rates, ease of photo-autotrophic cultivation, unique metabolic properties and continuing progress in algal transgenics have raised interest in the use of microalgae systems for recombinant protein (RP) production. This work demonstrates the development of an advanced RP production and secretion system for the green unicellular model alga *Chlamydomonas reinhardtii*. We generated a versatile expression vector that employs the secretion signal of the native extracellular *C. reinhardtii* carbonic anhydrase for efficient RP secretion into the culture medium. Unique restriction sites were placed between the regulatory elements to allow fast and easy sub-cloning of sequences of interest. Positive transformants can rapidly be identified by high-throughput plate-level screens via a coupled *Gaussia* luciferase marker. The vector was tested in *Chlamydomonas* wild type CC-1883 (WT) and in the transgene expression transformant UVM4. Compared to the native secretion signal of the *Gaussia* luciferase, up to 84% higher RP production could be achieved. With this new expression system we could generate transformants that express up to 10 mg RP per liter culture without further optimization. The target RP is found exclusively in culture medium and can therefore easily be isolated and purified. We conclude that this new expression system will be a valuable tool for many heterologous protein expression applications from *C. reinhardtii* in the future.

1. Introduction

The study of genetic modification has come far from the first understanding of chemical and radiation induced mutagenesis (Auerbach and Robson, 1944; Demerec and Latarjet, 1946; Witkin, 1947) and the first successful transformation of a foreign vector into a bacterial host (Cohen et al., 1973) to the current wealth of knowledge available to researchers. Since the advent of foreign protein production in *Escherichia coli* (Itakura et al., 1977) the idea-scape surrounding RP expression has exploded into a vibrant mosaic of host organisms, targets, and strategies. Today, recombinant expression systems exist for species from most kingdoms while transformation strategies and host ranges are accelerating rapidly with the advent of flexible synthetic DNA technologies. Chimeric gene expression techniques are valuable for functional analysis of cellular mechanics, modifying traits of host organisms, and the production of high value bio-products. Genetic manipulation is possible in many bacterial (Sahdev et al., 2008; Terpe, 2006), mammalian (Chu and Robinson, 2001; Schmidt, 2004), plant (Fischer et al., 2004; Manuell et al., 2007), insect (Ikonomou et al., 2003), yeast (Porro et al., 2005), and fungal (Talabardon and Yang, 2005) systems.

Bacterial hosts are widely used for RP production (Terpe, 2006), however, lack eukaryotic post translational modification capabilities and may require complicated processing steps to handle purification of proteins from inclusion bodies (Sahdev et al., 2008). Yeast based systems present an interesting alternative to prokaryotic expression systems, demonstrating basic eukaryotic machinery, known genetics, ease of handling, and the capacity for protein secretion (Porro et al., 2005). However, yeasts maintain species specific post-translational modification and secretion mechanisms often result in insufficient protein yields (Porro et al., 2005). Insect cell expression systems also maintain variable post-translational modification properties and can be limited by costs and technical difficulties of scale-up as well as the inherent lytic nature the baculovirus-based system (Ikonomou et al., 2003).

Abbreviations: ALG, asparagine linked glycosylation; CAH1, *Chlamydomonas reinhardtii* carbonic anhydrase 1; *c*CA, secretion signal of CAH1; CHO, Chinese hamster ovary; gLuc, Gaussia princeps luciferase; *Lp*IBP, *Lollium perenne* ice binding protein; PTM, posttranslational modification; RP, recombinant protein; TAP, Tris acetate phosphate; TSP, total soluble protein.

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Although fungal systems are generally good at production of fungal enzymes, inherent high concentration of proteases and technical limitations in scale-up have limited this system as an expression host to a few targets (Punt et al., 2002).

Posttranslational modifications (PTMs), specifically, nitrogen (*N*-) and oxygen (*O*-) linked glycosylation motifs are of great importance to RP production due to the effect these motifs have on biological and immune activity *in vivo* (Gomord et al., 2010). In order to develop similar glycosylation to native human patterns, which control bio-compatibility and bio-activity of therapeutic protein targets, many therapeutic RPs are produced in mammalian cell lines. Some commonly employed cell lines include baby hamster kidney (BHK) (Schmidt, 2004), Chinese hamster ovary (CHO) and mouse murine myeloma lines SP2/0 and NS0 (Chu and Robinson, 2001). These systems have drawbacks of low protein yields and expensive cultivation often requiring complex media containing serum and growth factors, although serum-free media are becoming common (Chu and Robinson, 2001).

Plant based expression systems have emerged as a platform for RP production, specifically therapeutic proteins (Fischer et al., 2004). So-called molecular farming leverages the high biomass yields of crop plants with potentials of creating edible biologics for applications such as vaccination (Yu and Langridge, 2003).

Of the plant based cell systems, unicellular microalgae have recently gained interest as hosts for RP production due to their relatively rapid growth rates in comparison to plant expression systems, favorable transformation turnaround time, ease of containment, scalability, as well as inexpensive cultivation in simple media close in composition to water (Franklin and Mayfield, 2004; Mayfield et al., 2007; Specht et al., 2010). Of the eukaryotic microalgae, the photosynthetic model organism *Chlamydomonas reinhardtii* is largely targeted for genetic manipulation strategies. The genus *Chlamydomonas* has a long history of functional mutation studies as a model photosynthetic organism (Lewin, 1952). Coupled with ease of *C. reinhardtii* transgene insertion (Kindle, 1990) and fully sequenced nuclear, chloroplast, and mitochondrial genomes (Specht et al., 2010), this microalgae is an attractive species for RP technologies. To date, viable RP production has focused on chloroplast transgene expression in *C. reinhardtii* (Bateman and Purton, 2000) as the plastid genome is amenable to efficient homologous recombination, has well described regulation of gene expression (Herrin and Nickelsen, 2004), and has demonstrated protein yields up to 5% of total soluble protein (TSP) (Manuell et al., 2007). In contrast, nuclear gene expression in *C. reinhardtii* has demonstrated comparably low yields of target RPs. Through mutagenesis strategies a strain of *C. reinhardtii* has been engineered which is able to reach 0.2% TSP from nuclear gene expression (Neupert et al., 2009), however, this pales in comparison to chloroplast based strategies. Indeed over the last ten years a great deal of information has been elucidated regarding the regulation of nuclear transgene expression in this organism, such as the requirement for codon optimization to match the ~62% GC content of the *C. reinhardtii* nuclear genome (Ruecker et al., 2008; Shao and Bock, 2008). Optimized promoter sequences and organism specific regulatory elements have also been identified (Schroda et al., 2000), and some reports have demonstrated that nuclear gene expression can be further improved by masking the sequence to be expressed in introns of native genes (Eichler-Stahlberg et al., 2009; Lumbreras et al., 1998).

A common strategy in industrial RP production is targeted secretion of RPs to the periplasmic or extracellular space (Fischer et al., 2004; Porro et al., 2005; Schmidt, 2004). It is considered that protein localization into the periplasmic space has reduced exposure to protease activity, less inhibitory feedback to gene expression processes, and can allow continuous cultivation in perfusion-style bioreactors. Proteins that are secreted through the ER and Golgi exocytosis pathway are also subjected to PTMs (Doran, 2000; Eichler-Stahlberg et al., 2009; Kukuruzinska and Lennon, 1998; Porro et al., 2005; Punt et al., 2002). Although not confirmed by other reports, a recent report has claimed that *C. reinhardtii* contains sialylated N-glycans similar to mammals, a feature not found in other plant expression systems (Mamedov and Yusibov, 2011). As PTMs are not performed on proteins produced within the chloroplast (Mayfield et al., 2007), the production of nuclear encoded genes targeted for secretion may provide a means of increasing protein production as well as new avenues for RPs depending on target PTM motifs.

In light of developments in synthetic biology, we constructed an expression vector *de novo* from known regulatory elements for *C. reinhardtii* nuclear gene expression (Heitzer and Zschoernig, 2007). Certain markers have been demonstrated with a capacity for secretion outside of the *C. reinhardtii* cell (Eichler-Stahlberg et al., 2009; Ruecker et al., 2008; Shao and Bock, 2008). We postulated that the replacement of predicted secretion signal of the marker luciferase protein from *Gaussia princeps* (*g*Luc) with that of a native *C. reinhardtii* extracellular enzyme carbonic anhydrase 1 (CAH1) (Fukuzawa et al., 1990) would improve transgene localization to the culture media and perhaps RP abundance. This manuscript outlines the combination of synthetic biology, plate level protein abundance assay and RP quantification strategies used to demonstrate robust nuclear gene expression and extracellular localization from transformant *C. reinhardtii* cells.

2. Materials and methods

2.1. Vector construction

To create vector pgLUC, the sequence of plasmid pHsp70A/RbcS2-cgLuc (Fuhrmann et al., 2004; Heitzer and Zschoernig, 2007) between the restriction endonuclease sites *Sac*I and *Kpn*I was modified in silico to contain a codon optimized version of the *Gaussia princeps* luciferase (*g*Luc) marker with C-terminal *Lollium perenne* ice binding protein (Middleton et al., 2012; Sidebottom et al., 2000) (*Lp*IBP) and hexa-histidine tags. Unique restriction digest sites were inserted between each regulatory and gene element. The sequence of *Bgl*II was inserted in the coding region immediately after the *g*Luc predicted N-terminal secretion signal determined by SignalP server (Petersen et al., 2011). The entire cassette was synthesized by GeneArt (Life Technologies) via oligonucleotide annealing. This 1.8 kb fragment was sub-cloned into vector pJR38 (Neupert et al., 2009) between restriction endonuclease sites *Spe*I and *PshA*I to incorporate its paromomycin resistance cassette. DNA was digested with FastDigest® restriction endonucleases, run on a 2% agarose gel at 120 V for 35 min and visualized with SYBR® Safe DNA Gel Stain (Life technologies). Purified linear fragments were ligated with Quick T4 DNA Ligase (NEB) and transformed into Dh5α *Escherichia coli* cells by heat shock.

Vector pcCAgLUC was designed to contain the *C. reinhardtii* carbonic anhydrase 1 (Fukuzawa et al., 1990) (CAH1) secretion signal (*c*CA) in place of the predicted secretion signal of the *g*Luc. The *c*CA sequence was amplified by polymerase chain reaction (PCR) using the primers FW_{NdeI} 5′ aatt**catatg**GCGCGTACTGGCGCTCT 3′, RV_{BglII} 5′ aatt**agatct**AGCCTGCGCGCAGC 3′ from a CAH1 containing plasmid template. PCR was cycled with: 95 °C for 2:00 min, 35 cycles of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 15 s, followed by 72 °C for 8:00 min. Reactions resulted in a single visible band of approximately 60 bp that was processed as above. This fragment and pgLUC, were digested with *Nde*I and *Bgl*II and processed through to ligation as above.

Vector pNSgLUC was created through amplification of the codon optimized *g*Luc sequence omitting its N-terminal targeting region