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REVIEW

Chlamydomonas reinhardtii as a viable platform for the production of recombinant proteins: current status and perspectives

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Abstract *Chlamydomonas reinhardtii* has many advantages compared with traditional systems for the molecular farming of recombinant proteins. These include low production costs, rapid scalability at pilot level, absence of human pathogens and the ability to fold and assemble complex proteins accurately. Currently, the successful expression of several proteins with pharmaceutical relevance has been reported from the nuclear and the chloroplastic genome of this alga, demonstrating its usefulness for biotechnological applications. However, several factors affect the level of recombinant protein expression in *Chlamydomonas* such as enhancer elements, codon dependency, sensitivity to proteases and transformation-associated genotypic modification. The present review outlines a number of strategies to increase protein yields and summarizes recent achievements in algal protein production including biopharmaceuticals such as vaccines, antibodies, hormones and enzymes with implications on health-related approaches. The current status of bioreactor developments for algal culture and the challenges of scale-up and optimization processes are also discussed.

Keywords Microalgae · Biopharmaceuticals · Bioreactor · Protein yield

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Introduction

Microalgae have the potential to revolutionize biotechnology since they have many features that are desirable as a commercial recombinant protein expression system, including high growth rate and ease of cultivation, with the ability to perform post-transcriptional and post-translational modifications (Surzycki et al. 2009). Microalgae also exhibit superior photosynthetic efficiency, being approximately three times more efficient in using light than higher plants (Shimizu 1996). Furthermore, microalgae contain large amounts of proteins ranging from 50 to 70% of the fresh weight. This is a much higher percentage than the choicest edible parts of any higher plant or animal (Passwater et al. 1997).

Another feature which facilitates rapid progress in developing transgenic microalgae strains is that there are well-established transformation methodologies for chloroplast and nuclear genomes, with a relatively short time required between the generation of initial transformants and the assessment of protein expression (Boynton et al. 1988; Harris 1989; Franklin and Mayfield 2004). Additional benefits include the ability to produce secreted proteins and the availability of a wide variety of well-characterized chloroplast and nuclear promoter elements (Rochaix 1996; Rochaix et al. 2000).

Besides the fact that most green algae are classified as "Generally Regarding As Safe" (GRAS), they can be cultivated in full containment reducing any concern about environmental contamination. These characteristics make these systems an attractive approach for the expression of recombinant proteins (Mayfield and Franklin 2005; Mayfield et al. 2007).

To date, the large majority of current work is performed with *Chlamydomonas reinhardtii*, as it is the best characterized of the microalgal species. Transformation of the *C. reinhardtii* chloroplast genome was demonstrated 20 years ago (Boynton et al. 1988), and is achieved by site-specific recombination between homologous DNA sequences present in the construct and target plastome (Newman et al. 1990). *C. reinhardtii* encloses a single large chloroplast that occupies approximately 40% of the cell volume. That chloroplast contains its own genome, which is a circular molecule of approximately 200 kb, and each chloroplast contains approximately 80 identical copies of the genome. Consequently, stable transformation of the chloroplast requires that all 80 copies convert to the recombinant form. In contrast to the typical nuclear transformation, plastid transformation is frequently performed via homologous recombination; hence, integration events can be precisely targeted to any region in the chloroplast genome that contains a so-called "silent site" for transgene integration (Newman et al. 1990; Goldschmidt-Clermont 1991).

The continuing development of genetic engineering tools for this microalgae has allowed the expression of recombinant vaccines (Dreesen et al. 2010), fully functional antibodies (Franklin and Mayfield 2005; Tran et al. 2009), other therapeutics (Boehm 2007; Weathers et al. 2010), and proteins of biotechnological relevance (Li and Tsai 2009; Voigt et al. 2004) at economically viable levels. However, many obstacles remain to be overcome before microalgae can be considered standard expression systems. The aim of this review is to examine the factors affecting protein expression and strategies for its mainstream implementation; it also describes current successes in algal protein production and provides an outlook for the future of microalgal biotechnology.

Factors affecting protein expression and strategies for its increase

Promoters, 5′ and 3′UTR sequences

One of the main objectives in molecular farming is the production of recombinant proteins at high yields. To achieve high yields, expression-construct design must optimize all stages of gene expression, from transcription to protein stability; therefore, the expression constructs in which the transgene is bracketed must include various regulatory elements that are known to be optimal in microalgae.

For high-level transcription, the most important elements are the promoters and 5′/3′ regulatory mRNA untranslated regions (UTRs).

The PsaD, RbcS2 and HSp70A promoters have been widely used to drive nuclear gene expression in *C. reinhardtii* and they are currently considered the best constitutive promoters available (Schroda 2006; Fischer and Rochaix 2001; Kindle 1998). In chloroplast of *C. reinhardtii*, the rbcL, atpA, psbD and psbA promoters and their respective UTRs are now the most popular choice (Manuell et al. 2007; Surzycki et al. 2009).

Nuclear promoters

The most widely used constitutive promoter in *C. reinhardtii* has been that from RbcS2, which has been used to express a non-acetylable α-tubulin, reaching a fivefold overexpression compared to the TubA promoter (Kozminski et al. 1993). The RbscS2 promoter has since been used in constructs to express the *Cry1*, *ble* and *aadA* genes (Cerutti et al. 1997; Stevens et al. 1996; Nelson et al. 1994). However, other constitutively active promoters are shown to be highly active in chimeric constructs (see Table 1).

In 2001, Fischer and Rochaix created a new nuclear expression vector for *C. reinhardtii* using a genomic fragment containing the *PsaD* gene. This vector allows an efficient expression of the *Ble* and *Arg* genes, which serve as endogenous and exogenous selectable markers, respectively. According this report, the main advantage of the PsaD is that the full regulatory sequence appears to lie within its promoter and 5′ and 3′ untranslated regions. In addition, this vector is particularly useful for gene expression as it does not require the time-consuming insertion of introns to enhance the expression.

Synthetic promoters have also been developed by fusing the Hsp70A (heat shock protein 70A) promoter to other *Chlamydomonas* nuclear promoters (Schroda et al. 2000). In that study, the authors demonstrated that the Hsp70A promoter serves as a transcriptional enhancer of promoters RbcS2, β2-tubulin and Hsp70B leading to improved transgene expression in *Chlamydomonas* under inducing conditions. Additionally, these promoter fusions exhibited new regulatory properties. Furthermore, it was demonstrated that the transformation efficiency is significantly increased when the Hsp70A promoter is fused upstream to the RbcS2 promoter, driving the expression of the *aadA* cassette (Schroda et al. 2000).

Another relevant strategy in this field has been focused in the design of *Chlamydomonas* strains that efficiently

Table 1 Nuclear promoters of *Chlamydomonas reinhardtii* used for the expression of heterologous proteins

Promoter	Gene source	Reference
RbcS2	Ribulose bisphosphate carboxylase small subunit	Kindle (1998)
TubA1	Alpha-tubulin	Kozminski et al. (1993)
Pcy1	Plastocyanin	Quinn and Merchant (1995)
AtpC	Gamma-subunit of chloroplast ATPase	Quinn and Merchant (1995)
β-tub	β-Tubulin	Blankenship and Kindle (1992)
PsaD	Photosystem I complex	Fischer and Rochaix (2001)
Hsp70A	Heat shock protein 70A	Schroda et al. (2000)

express nuclear transgenes, which consists in introducing mutations that prevent the epigenetic transgene suppression mechanism. Neupert et al. (2009) generated several UV strains mutants. Among these, the UVM4 and UVM11 strains showed the highest levels in transgene expression, probably because these strains contain a mutation within an endogenous gene which encodes a regulator of chromatin structure.

Chloroplast promoters

Previous works have identified regions within the 5′UTR that can dramatically impact on mRNA stability and potentially be used to increase mRNA stability as a means to increase recombinant protein synthesis (Salvador et al. 2004; Suay et al. 2005). This is achieved probably through interaction of endogenous UTRs and coding regions which results in secondary structures that enhance translational efficiency by interactions with trans-acting protein factors (Vaistij et al. 2000; Eberhard et al. 2002; Franklin and Mayfield 2004). Some studies have revealed that translational efficiency is a rate-limiting step for chloroplast gene expression and have shown that the 5′UTRs of plastid mRNAs contain key elements for translational regulation (Gallie 1998; Wells et al. 1998; Eberhard et al. 2002; Nickelsen 2003).

In 2005, Barnes et al. assessed the effect of various endogenous UTRs on recombinant protein expression in *C. reinhardtii* chloroplasts. Using the promoters and 5′UTR of five *C. reinhardtii* chloroplast genes, *atpA*, *rbcL*, *psbA*, *psbD* and *16S rRNA*, fused to a codon-optimized *gfp* reporter followed by the 3′-UTR of either the *atpA, rbcL, psbA* or $tRNA_{arg}$ genes, they obtained a series of chimeric genes which were integrated in the chloroplast genome. The examination of mRNA accumulation from this set of reporter constructs demonstrated that each of the different promoters and 5′/3′-UTRs constrain the accumulation of chimeric mRNAs at very different levels. They observed that the protein accumulation was in general proportional to the mRNA accumulation, obtaining the highest levels of recombinant protein using both the atpA or psbD promoters and 5′UTRs; a much lower protein accumulation was observed under control of *rbcL* and *psbA*, even no expression using the 16S rRNA 5′UTR was detected (Barnes et al. 2005). In a recent work, Rasala et al. (2010) reported that psbA and atpA chloroplast promoters support the expression of three recombinant proteins comprising 14FN3, VEGF and HMGB1. Interestingly, the psbA promoter along with the corresponding UTRs results in a 20-fold increase on protein accumulation in comparison to those levels reached by constructions carrying the atpA promoter and its 5′UTR (Table 2).

Also, in 1999, Eibl et al. reported that efficient translation of chimeric transcripts in tobacco chloroplast is dependent on the 5′ leader rather than the 3′UTR. However, it has been proposed that the RNA secondary structures in the 3′UTR have functions in mRNA stabilization, 3′ end elongation, transcription termination, and transcripts accumulation in *C. reinhardtii* chloroplasts (Stern et al. 1991; Blowers et al. 1993; Rott et al. 1998).

Kasai et al. (2003) using a non-photosynthetic rbcL-deficient strain (mutant DEVL) have demonstrated that using atpA and psbD chloroplast promoters and 5′-UTR in chimeric constructs did not affect heterologous gene expression, employing a translational fusion comprising the uidA reporter gene and the coding region of either *atpA* and *psbD* gene. No significant changes were observed in the expression of these fusion proteins in comparison to the native genes. In contrast, other chimeric constructs containing psbA and rbcL promoters along with the corresponding *psbA* and *rbcL* genes, showed more dependence on the unrelated fused sequence (Table 2). A possible explanation for this is that the *rbcL* and *psbA* UTRs interact with the GFP or GUS coding regions in such a way as to limit translation of these chimeric mRNAs. It is possible, for example, that these heterologous coding sequences may prevent the formation of RNA secondary structures that are recognized by translation factors or favoring the ribosome binding at the initiation codon (Barnes et al. 2005). In a recent work, however, Rasala et al. (2011) reported high levels of heterologous protein accumulation using the psbA

Table 2 Chloroplast promoters of *Chlamydomonas reinhardtii* used for the expression of heterologous proteins

Promoter	Gene source	Highest expression level achieved (% TPS)	Construct (prom-gene-3′UTR)	Strain	Reference
atpA	Alpha subunit of adenosine triphosphatase	~3–4	atpA-CTBVP1-rbcL[a]	137c (mt+)	Sun et al. (2003)
psbD	Photosystem II D1	~0.25	psbD-M-SAA-psbA[b]	137c (mt+)	Manuell et al. (2007)
rbcL	Ribulose bisphosphate carboxylase large subunit	~0.7	rbcL-CTBD2-rbcL[c]	125c (mt+)	Dreesen et al. (2010)
psbA	Photosystem II psbA	~3	psbA-14FN3-psbA[d]	137c (mt+)	Rasala et al. (2010)

[a] Fusion protein comprising foot-and-mouth disease virus VP1 protein and the cholera toxin B subunit (CTBVP1)

[b] Mammary-associated serum amyloid (M-SAA)

[c] Fusion protein comprising the D2 fibronectin-binding domain of *Staphylococcus aureus* and cholera toxin B subunit (CTBD2)

[d] Domain 14 of human fibronectin (14FN3)

promoter/5′UTR but only in a psbA-deficient genetic background.

Other report that supports the critical role of the translational regulation was published by Coragliotti et al. (2011) in which it is speculated that some recombinant mRNAs have sequences that impart a general disability to recruit translation factors, independently of the light conditions. A careful analysis of all translation steps was therefore recommended, including the study of the nature of potential 5′UTR-coding region interactions and the identification of trans-acting factors that impact translation of chloroplast mRNAs at different levels.

One strategy to be explored in chloroplast of *C. reinhardtii* is the use of the 5′UTR from the phage 7 fused with the first ten aminoacids of the N-terminal of the coat protein gene of potato virus X, which has been demonstrated in other systems such as plants and bacteria, to be useful in obtaining the highest expression levels (Hefferon et al. 2000; Herz et al. 2005).

All of these data suggested that the translation and mRNA accumulation are determined primarily by the promoter and 5′UTR; therefore, the choice of promoter and regulatory regions are critical factors to consider for each protein of interest in order to achieve high yield of recombinant proteins.

Codon dependency

The accumulation of transgenic protein depends not only on the transcription rate of its coding gene, determined principally by the promoter and UTR sequences used, but also on the steady-state level of its mRNA, efficiency and translation rate of the mRNA and stability of the protein. Expression in heterologous systems has demonstrated the importance of altering codon usage in order to increase the rate of protein production. Transgenes from heterologous species often have a different codon bias to the host cell, which might result in pausing at disfavoured codons and truncation, misincorporation or frameshifting (Campbell and Gowri 1990) and also may decrease their susceptibility to silencing (Heitzer et al. 2007).

The chloroplast of *C. reinhardtii* displays such codon bias, with codons containing adenine or thiamine nucleotides in the third position favored over those with guanine or cytosine (Nakamura et al. 1999). Several works have confirmed the impact of altering the codon composition of a transgene to obtain an increase upon recombinant protein accumulation in *C. reinhardtii* chloroplasts. A codon-optimized green fluorescent protein reporter gene (*gfp*) and non-optimized native *gfp* genes, both driven by the rbcL promoter and 5′UTR, were transformed into chloroplasts and the accumulation of the recombinant protein was evaluated. The quantitation of GFP accumulation showed that the codon-optimized *gfp* gene resulted in an 80-fold increase in GFP accumulation compared with the non-optimized version (Franklin et al. 2002). Similar studies using a codon-optimized human antibody gene (Mayfield et al. 2003) and codon-optimized luciferase genes (*luxAB* and *luxCt)* (Mayfield and Schultz 2004) confirmed that codon bias plays a significant role in protein accumulation in *C. reinhardtii* chloroplasts.

The Codon Adaptation Index (CAI) is frequently used as a quantitative method to predict the expression level of native and heterologous genes based on their codon usage (Xia 2007). As the chloroplast, mitochondrial and nuclear genomes of *C. reinhardtii* may exhibit different codon biases, therefore genome-specific CAI values should be obtained for optimal translational (Potvin and Zhang 2010). The analysis of codon usage can be obtained using free software recently developed to estimate CAI values. One of these tools is the E-CAI (http://genomes.urv.es/CAIcal/E-CAI), which is useful to determining if differences in CAI between sequences are significant (Puigbo et al. 2008). The OPTIMIZER application (http://genomes.urv.es/

OPTIMIZER) is helpful to optimize the codon usage (Puigbo et al. 2007).

Consequently, codon optimization is an effective and necessary step in gene sequence optimization, and a relatively simple way to address due to the recent advances in DNA synthesis technology.

Transformation-associated genotypic modifications

Since transgenic strains are usually obtained by biolistic transformation using a single chloroplast integration vector and inserted into chloroplast DNA (cpDNA) by homologous recombination, it is thus expected that each obtained transformant, including the level of recombinant protein, should be identical; however, in 2009, Surzycki et al. reported a range of protein accumulation in chloroplast varying from 0.88 to 20.9% of total cellular protein (TCP) in pBA155 transformants. The authors suggested that the expression of recombinant proteins may depend to a lesser extent on the promoter, site of insertion or associated 3′ and 5′UTRs than to the nature of changes incurred during an individual chloroplast transformation event. They associated this variation to genotypic modifications resulting from the transformation process, each one referred as transformosomes, leading to transformed lines having unique characteristics.

Although the mechanism behind transformosomes is unknown, they may be due to additional insertion of the transgene into the chloroplast genome target site and also the nuclear genome, which can include additional insertions of the vector or DNA fragments that are able to affect the function of nuclear gene(s) which would interfere with proteins regulating recombinant yields. Nuclear insertions might also interfere with the expression of chloroplast-bound genes necessary for photosynthesis or with proteins implicated in their targeting and transfer. Another additional hypothesis is that regulation of the steady-state level of the mRNA is affected, which might influence the levels of mRNA present in each transformant (Surzycki et al. 2009). Hence, a large population of transformed cells should be screened to isolate the most productive clone. However, analysis by Southern blot has not been performed to demonstrate or to dismiss the influence of nuclear insertions in the transformosomes.

Influence of intron sequences as endogenous enhancers

Even though the introns commonly are not encoding protein sequences, they play an important part in genome-wide processes such as exon shuffling (Liu and Grigoriev 2004). Also, the introns can affect the expression of distinct genes by alternative splicing or by regulation of transcription (Liu et al. 1995; Lareau et al. 2004). It has been shown, as in other organisms including plants and other microalgae, that inserting introns from native genes in heterologous sequences result in increased protein yields (Koziel et al. 1996; Lumbreras et al. 1998; Berthold et al. 2002).

In 2009, Eichler-Stahlberg et al. demonstrated, in a nuclear expression strategy, that the random insertion of at least one intron into the RBCS2 gene resulted in a fourfold increased expression, compared to base levels of a *Renilla*-luciferase gene used as a reporter. Although none any of the three RBCS2 introns alone had a positive effect on nuclear expression, their integration in their physiological order and number created an over-proportional stimulating outcome observed in all transformants, which implies an endogenous enhancer element (Lumbreras et al. 1998; Berthold et al. 2002).

The mechanism of how the introns increase transgene expression is unclear, but it is thought that they might act at a transcriptional or post-transcriptional level. In a transcriptional level, the activation might be reflected by increased de novo synthesis of RNA as a result of a direct stimulation of the initiation of RNA-polymerase II or even as an indirect effect by the prevention of gene silencing (Lumbreras et al. 1998; Schroda 2006). In a post-transcriptional level, the positive influence might be related to enhanced RNA maturation initiated by the additional splicing event (Bentley 2005). Therefore, another option to achieve high yields of recombinant proteins may include, in the expression construct, intron sequence as endogenous enhancers.

Protein localization and sensitivity to proteases

One of the primary factors affecting the yield of recombinant proteins is the proteolysis. Proteolytic enzymes are essential for endogenous protein processing, however, they can degrade foreign proteins after elongation, or interfere with their correct assembly and post-translational modification. Several chloroplast proteases have been characterized in recent years, all of these are homologous to bacterial proteases and are energy dependent (Adam et al. 2006). Similar to their bacterial homologous, these proteases are involved in the removal of defective or abnormal native proteins. Proteolysis may also lead to inconsistent results and to difficulties in downstream processing or purification due to degraded or non-functional protein fragments (Preiss et al. 2001). It has previously been demonstrated that energy-dependent proteases in *C. reinhardtii* are also involved in proteolytic degradation of foreign proteins (Surzycki et al. 2009).

Considering that each target protein behaves individually as a consequence of several factors, including the rate of proteolysis, some attempts have been drawn in

order to minimize the proteolytic degradation of recombinant proteins, even though mainly efforts have been developed for plants, they may also be applicable for microalgal systems. For nuclear-expressed proteins, degradation can be minimized by targeting protein synthesis to the endoplasmic reticulum (ER) rather than to the cytosol which could be achieved using an H/KDEL C-terminal tetrapeptide tag in the expression construct (Conrad and Fiedler 1998; Ma et al. 2003). Using an ER-retention tag, the yields are generally 2-fold to 10-fold greater (Conrad and Fiedler 1998), although another work has reported as far as 104-fold increase in tobacco plants (Wirth et al. 2004). The oxidizing environment of the ER, the lack of proteases and the abundance of molecular chaperones are important factors for proper folding and stability of certain proteins (Britt and May 2003). Additionally, some authors have explored the option of co-expressing protease inhibitors as a useful tool to increase the recombinant protein yields in plants, without disturbing normal growth and development (Van der Vyver et al. 2003).

Another possible strategy to minimize proteolytic degradation of proteins requiring post-translational modification would consequently consist of targeting the nuclear-expressed proteins to the chloroplast for storage (Jarvis 2008). However, if the protein does not require post-translational modifications it can be expressed directly in the chloroplasts of algae (Bock 2001) with the advantage of having a more sheltered environment with less proteolytic activity.

Inducible gene expression system in *C. reinhardtii*

There are few studies about the development of controllable gene expression systems in *C. reinhardtii*. In this context, some chemically regulated promoters have been described, among which are the nitrate reductase (NIT1) promoter induced by ammonium starvation (Ohresser et al. 1997); the carbonic anhydrase (CAH1) promoter induced by low CO_2 (Kucho et al. 1999); and the cytochrome C6 (CYC6) promoter induced by copper (Cu) depletion or nickel (Ni) addition (Searle et al. 1985; Serfling et al. 1985; Buikema and Haselkorn 2001; Quinn et al. 2003; Ferrante et al. 2008).

Additionally, it was observed in *C. reinhardtii* that short sequences in mRNAs called riboswitches, such as *THI4* riboswitch (Croft et al. 2007) and the Cyc6–Nac2 system (Surzycki et al. 2007), can negatively regulate the foreign-level expression by adding thiamine or copper to the growth medium. These inducible systems have interesting implications when the regulation of protein levels is required due to its physiological role or when the heterologous protein has a toxic effect in the host.

On the other hand, Kato et al. (2007) constructed two artificial control systems in *C. reinhardtii* using the lac regulation systems from *Escherichia coli*; however, these systems failed both in induction and repression. Additionally, IPTG is required as inducer, which is expensive thus rendering the system less feasible for industrial applications. However, there are other inducible systems in *E. coli* that could be assayed on *C. reinhardtii* for developing a controllable expression system in a cost-effective manner. These systems could be based on an osmotically signaled transcription activation using *pro U* promoter (Mellies et al. 1994), or by cold-shock activation utilizing the promoter derived from *cspA* gene, a cold-shock gene of *E. coli* (Goldenberg et al. 1997). The implementation of these systems will allow the temporal or developmental control for the expression of specific genes, thus facilitating the precise determination of their function or the precise expression control of the expression of potentially toxic proteins, for industrial or pharmaceutical uses. Then, using inducible instead constitutive promoters will allow the expression of potentially toxic proteins only after reaching high cell densities and therefore optimizing protein yields.

Polycistronic expression in *C. reinhardtii*

Production of recombinant proteins in *C. reinhardtii* chloroplast offer some advantages such as high accumulation of recombinant protein and transcription of multiple genes using the same promoter (polycistronic) or its availability for multiple insertion sites. Then, reconstitution of multi-subunit proteins or metabolic pathways can be done in transgenic *C. reinhardtii* chloroplasts in a single transformation event. For example, Su et al. (2005) inserted both, large and small subunits of allophycocyanin gene (*apcA* and *apcB*) into *C. reinhardtii* chloroplast and the expressed foreign proteins in transformants accounted for around 2–3% of total soluble protein (TSP). Also, rather than expressing single enzymes to alter metabolic fluxes, it will most likely be required to coordinate the expression of all members of a metabolic "channel" to obtain the desired changes (Hunt and Maiti 2001). Furthermore, on vaccine development field it would provide the ability to produce the antigen of interest along with adjuvants, or even multicomponent formulations with broader immune coverage. Since the expression of more than one open reading frame can be achieved using bi or polycistrons, the chloroplast of this algae is a suitable system to produce multiprotein complexes such as enzymes, antibodies, receptors, ion channels and other multi-subunit proteins or to promote the proper folding and assembly of antibodies by means of co-expressing chaperonins. This would have profound implications on developing transformed lines, useful for the biotechnological approaches.

Fusion to an endogenous protein

The fusion of recombinant products to native proteins has also resulted in an increase of protein yield. In 2009, Muto et al. reported that the fusion of a recombinant protein through a cleavable domain to an endogenous algal photosynthetic protein can increase the recombinant protein accumulation. The luciferase gene was fused at the carboxy-terminal end of the large subunit of ribulose bisphosphate carboxylase (Rubisco LSU) and a substantial increase (33-fold) in luciferase accumulation compared to the luciferase gene expressed alone was observed. These results indicate that recombinant protein accumulation in algal chloroplasts can be improved by fusion with a native protein.

Current developments in microalgal bioreactor systems

Algae-based candidate vaccines

A candidate vaccine against type I diabetes

Although the etiology of type I diabetes is multifactorial, it is known that autoreactive T cells infiltrate the pancreas and are targeted against multiple autoantigens, including the glutamic acid decarboxylase (GAD)3 and insulin, which are known to be major β-cell autoantigens that play an important role in the development of type I diabetes (Bach and Chatenoud 2001) and therefore might have implications in the development of novel antigen-specific therapies for the treatment of type I diabetes. Since recombinant production of hGAD65 by means of conventional bacterial or mammalian cell platforms present low yield and low efficiency Wang et al. (2008) performed the chloroplast transformation of *C. reinhardtii*. The full-length *hGAD65* gene, along with the *C. reinhardtii* chloroplast rbcL promoter and 5′/3′UTRs, were transferred to the chloroplast genome by particle bombardment. After proving the expression of the algal-derived hGAD65, it was able to react with sera from diabetic subjects and also to induce proliferation of spleen cells in Non-Obese Diabetic (NOD) mice, a commonly used murine model for the study of diabetes. Accumulation of the recombinant protein reached up to 0.25–0.3% TSP (Table 3). This approach demonstrates the potential of *C. reinhardtii* chloroplasts as a novel and attractive platform for the production of hGAD65 for immunization purposes (Wang et al. 2008).

A model for the foot-and-mouth disease virus vaccine

Foot-and-mouth disease (FMD) is an economically important and highly contagious disease of cloven-hoofed animals, most notably of cattle, pigs and sheep, but also several wild-life species (Alexandersen and Mowat 2005). FMD virus (FMDV) is capable of spreading rapidly in susceptible animals and thus FMD disease is serious enough to be monitored by the World Organization for Animal Health (OIE). FMDV is the prototype member of the *Aphthovirus* genus of the family Picornaviridae. The virus is antigenically highly variable and consists of seven serotypes (A, O, C, Asia1, SAT1, SAT2, and SAT3) and multiple subtypes (Grubman and Baxt 2004).

Sun et al. (2003) have reported the transferring of the pACTBVP1 vector to chloroplast genome of *C. reinhardtii* allowing the expression of a fusion protein comprising the foot-and-mouth disease virus (FMDV) VP1 and the cholera toxin B subunit (CTB), named CTBVP1. This design lies in the ability of the CTB protein to serve as a carrier and a potent mucosal adjuvant for unrelated antigens. Interestingly, the CTBVP1 accumulation levels reached up to 3% TSP and retained the GM1-ganglioside binding affinity, which is known to be associated to the adjuvant properties of this carrier (Table 2). The antigenicity of the two components was preserved, indicating a promising potential for this approach on the development of a mucosal vaccine against FMDV.

A highly effective Staphylococcus aureus vaccine

Staphylococcus aureus causes community-acquired and nosocomial infections and its natural habitat is the moist squamous epithelium in the anterior nares. About 20% of the human population carry *S. aureus* permanently in their nose and another 60% of individuals are intermittent carriers (Kluytmans et al. 1997). Infection rates are higher in carriers than in non-carriers and invasive disease is often caused by the patient's own isolate (Peacock et al. 2001).

The ability of *S. aureus* to cause infection is due, in part, to cell surface-associated proteins that mediate attachment to the host extracellular matrix termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Foster and Hook 1998).

Dreesen et al. (2010) engineered the chloroplasts of *C. reinhardtii* for the expression of the D2 fibronectin-binding domain of *S. aureus* as fusion with the cholera toxin B subunit (CTB), under the control of *rbcL* UTRs. The immunogenicity of the CTBD2 protein was assessed in mice proving that it was capable of inducing specific mucosal and systemic immune responses (Table 3). Moreover, the vaccination scheme significantly reduced the bacteria load in the spleen and the intestine of the immunized group and has been shown to provide 80% protection against lethal challenge of *S. aureus*. Interestingly, the alga biomass containing the vaccine was stable for more than 1.5 years at room temperature. These results underline the

Table 3 Biopharmaceuticals produced on *Chlamydomonas reinhardtii*

Biopharmaceutical	Type of biopharmaceutical	Expression strategy	Transformation technique	Expression levels	Assessed properties of the recombinant protein	Reference
Human glutamic acid decarboxylase 65 (hGAD65)	Vaccine against type I diabetes	Chloroplast	Biolistic	0.25–0.3% TSP	Reacts with sera from diabetic patients and is immunogenic in NOD mice	Wang et al. (2008)
Fusion protein comprising foot-and-mouth disease virus VP1 protein and the cholera toxin B subunit (CTBVP1)	Vaccine against foot-and-mouth disease virus	Chloroplast	Biolistic	3–4% TSP	Displays antigenic determinants from both components	Sun et al. (2003)
Fusion protein comprising the D2 fibronectin-binding domain of *Staphylococcus aureus* and cholera toxin B subunit (CTBD2)	Vaccine against *Staphylococcus aureus*	Chloroplast	Biolistic	0.7% TSP	Immunogenic in mice inducing mucosal and systemic immune responses when administered by the oral route. Reduces bacteria load and protects against lethal challenge with *S. aureus* at 80%	Dreesen et al. (2010)
Structural protein E2 from classical swine fever virus	Vaccine against classical swine fever virus	Chloroplast	Biolistic	1.5–2% TSP	Immunogenic in mice inducing systemic immune responses when administered by the s.c. route	He et al. (2007)
Fusion protein comprising the C-terminal domains from the Apical Major Antigen AMA1, or Major Surface Protein MSP1 and the granule-bound starch synthase (GBSS)	Vaccine against malaria	Nuclear	Glass beads	NR	Immunogenic in mice inducing systemic protective immune responses when administered by the oral and intraperitoneal routes	Dauvillée et al. (2010)
Unique large single-chain antibody against the glycoprotein D of the herpes simplex virus (hsv8-lsc)	Antibody for passive immunity	Chloroplast	Biolistic	≈1% TSP	Binds HSV proteins	Mayfield et al. (2003)
83K7C IgG1 human antibody	Antibody for passive immunity	Chloroplast	Biolistic	NR	Capable of binding and neutralize anthrax toxin	Tran et al. (2009)
Erythropoietin (EPO)	Treatment of anemia	Chloroplast	Biolistic	Very low	NR	Rasala et al. (2010)
Domain 10 of human fibronectin (10FN3)	Putative antibody mimic (monobody)	Chloroplast	Biolistic	Very low	NR	Rasala et al. (2010)
Domain 14 of human fibronectin (14FN3)	Putative antibody mimic (monobody)	Chloroplast	Biolistic	3% TSP	Functional assay not available	Rasala et al. (2010)
Human interferon b1	Treatment of multiple sclerosis	Chloroplast	Biolistic	ND	NR	Rasala et al. (2010)
Proinsulin	Treatment of type I diabetes	Chloroplast	Biolistic	Very low	NR	Rasala et al. (2010)
Human vascular endothelial growth factor (VEGF) isoform 121	Treatment of pulmonary emphysema and possible for erectile dysfunction and depression	Chloroplast	Biolistic	2% TSP	Active in a VEGF receptor-binding assay	Rasala et al. (2010)
High mobility group protein B1 (HMGB1)	Favors wound healing and potential as adjuvant for anticancer therapies	Chloroplast	Biolistic	2.5% TSP	Active in a fibroblast chemotaxis assay	Rasala et al. (2010)

%TSP percentage respect to total soluble protein, *NR* Not reported, *ND* Not detected

viability of developing an algae-based vaccine against this particular pathogen (Dreesen et al. 2010).

A vaccine approach against swine fever virus

He et al. (2007) have reported the expression of classical swine fever virus (CSFV) structural protein E2 in different chloroplast vectors, which has been shown to carry critical epitopes. Here, they reported a *C. reinhardtii* chloroplast expression vector, P64E2, containing classical swine fever virus structural protein E2 gene, which was constructed and transferred to *C. reinhardtii* by biolistic bombardment method. ELISA quantification assay showed that the expressed E2 protein accumulated up to 1.5–2% TSP (Table 2). The algae-derived E2 protein has been shown to be immunogenic by the subcutaneous route in mice. No significant responses were elicited when the antigen was administered by the oral route.

Malaria vaccine produced on starch granules

Malaria is by far the world's most important tropical parasitic disease, killing more children aged <5 years than any other disease, especially in Africa (WHO 2005). Malaria is caused by the protozoan parasite *Plasmodium*, which is transmitted by the bite of the female mosquito of any one of the 50 species of *Anopheles* mosquitoes, of which the best known is *A. gambiae* (Subbarao and Sharma 1997; Riehle et al. 2006). Four species of *Plasmodium* can cause human malaria: *P. falciparum*, responsible for the greatest number of deaths; *P. vivax*, which has the widest geographical distribution, *P. ovale* and *P. malariae* (Dubovsky and Rabinovich 2004). In the past 25 years, the development of an effective malaria vaccine has become one of the biggest riddles in the biomedical sciences. Experimental data using animal infection models demonstrated that it is possible to induce protective immunity against different stages of malaria parasites. Nonetheless, the vast body of knowledge has generated disappointments when submitted to clinical conditions and presently a single antigen formulation has progressed to the point where it may be translated into a human vaccine.

In an effort to explore the potential of an oral vaccine formulation against malaria, Dauvillée et al. (2010) expressed a nuclear-encoded protein comprising Plasmodium antigens fused to the granule-bound starch synthase (GBSS), which is the major protein associated to the starch matrix in all starch-accumulating plants and algae. The C-terminal domains from the Apical Major Antigen AMA1, or Major Surface Protein MSP1 were efficiently expressed as fusion proteins along with GBSS and bound to the polysaccharide matrix (Table 3).

An immunogenicity test was performed for the AMA1 in mice that were either immunized intraperitoneally with the engineered starch particles and Freund adjuvant, or fed with the engineered particles co-delivered with the mucosal adjuvant. Then mice groups were challenged intraperitoneally with a lethal inoculum of *P. berghei*. Both groups showed a significant diminished parasitemia with an extension of life span including complete cure for intraperitoneal delivery as assessed by negative blood thin smears. Moreover, it was proved that mice immunization with the *P. falciparum* GBSS–MSP1 fusion protein induced humoral responses able to inhibit in vitro the intraerythrocytic asexual development of *P. falciparum*, the most virulent plasmodial species. This approach based on the use of the so-called amylosomes opened a new avenue on the production of immunogenic forms of protective antigens in projects that aim to prove the concept of algae-derived vaccine. Further studies are required to determine if this approach enhances the immunogenicity in comparison with when it is produced as soluble free protein, effect that could be due to the high molecular weight complex formed or even the protection of the protein from degradation in the gut.

Perspectives in algae-based vaccine development

Microalgae chloroplast-derived vaccines have opened many prospects that could be helpful to adopt and develop strategies and methodologies for the production of vaccines. Some advantages to other chloroplast-derived vaccines can be mentioned. Regarding chloroplast-derived vaccines in plants, the expression of most vaccine antigens is still confined to tobacco. Although the use of tobacco for production of biopharmaceuticals proteins can be an alternative use for this plant, it cannot be used for oral administration because of its toxic alkaloid contents and therefore purification steps must be performed. Then, it is necessary to choose those plant species that can be consumed as raw material to fulfill the basic requirement of producing edible vaccines. However, the lack of efficient regeneration system for most edible plant species is the major limitation for extending the plastid transformation technology to other crops. On the other hand, *C. reinhardtii* chloroplast transformation has been well established in the last decade and high expression of proteins has been shown; additionally, this green microalgae fall into the GRAS category and allergens have not been reported so far (FDA 2002).

Among the relevant perspectives in this field, the need of improving immunogenicity is worth mentioning. One approach that remains to be extensively explored consists of co-expressing an adjuvant along with the antigen of interest. This goal is facilitated by targeting the expression of more than one open reading frame through polycistrons.

E. coli heat-labile enterotoxin B subunit (LTB), CTB or interleukins are interesting candidates since these have shown to improve the immunogenicity of a large number of vaccine formulations. Another alternative to assess would be the expression of new fusion proteins consisting of a carrying protein (CTB, LTB, VLPs) and the antigen of interest, which may result in facilitating the transport of the antigen from the gut lumen to the gut-associated lymphoid tissues and thus improving immunogenicity (Granell et al. 2010; Salyaev et al. 2010). Another strategy to explore includes the expression of a multi-epitope fusion protein in *C. reinhardtii* chloroplast (Soria-Guerra et al. 2011). In addition these perspectives would allow for the development of broad coverage vaccines.

Antibodies

Although antibodies constitute effective therapeutics against a variety of human diseases, the high cost of such biopharmaceuticals remains a limiting issue on their massive use. In a pioneering study, Mayfield et al. (2003) reported the efficient expression in the chloroplasts of *C. reinhardtii* of a unique large single-chain (lsc) antibody against the glycoprotein D of the herpes simplex virus (HSV). This contains the entire IgA heavy chain fused to the variable region of the light chain by a flexible linker. The synthetic gene was codon optimized in order to achieve high-level expressions of the expected hsv8-lsc protein in soluble form, which was detected by ELISA using HSV proteins prepared from virus-infected cells (Table 3). The dimerization by disulfide bonds was successfully carried out and it was possible to purify the hsv8-lsc antibody by the use of the flag epitope. These results opened a new perspective on antibody production in *C. reinhardtii*.

Interestingly, it was subsequently shown that *C. reinhardtii* was capable of synthesizing and assembling a full-length IgG1 human monoclonal antibody (mAb) into chloroplasts (Table 3). Tran et al. (2009) have published a report on the 83K7C antibody, derived from a human IgG1, which is directed against anthrax protective antigen 83 (PA83) and is able to neutralize the anthrax toxin in animal models. Heavy and light chain of 83K7C accumulated as soluble proteins that assemble into the expected quaternary complexes consisting of two heavy and two light chains. The algal-derived 83K7C showed a similar affinity to PA83 than that of the mammalian-derived counterpart. Other human IgG1 as well as a mouse IgG1 were expressed in *C. reinhardtii* as properly assembled antibodies (Tran et al. 2009). These findings support the viability of using algae to fold and assemble full-length human mAbs, which could lead to new cost-effective production platform of complex human therapeutic proteins.

Expression studies have demonstrated that many forms of recombinant antibody fragments can be functionally expressed in plants; these include the full-sized, chimeric and secretory IgG and IgA, single-chain Fv fragments (scFv), Fab fragments and heavy-chain variable domains (Hiatt et al. 1988; De Neve et al. 1993; Artsaenko et al. 1995; Baum et al. 1996).

Given that only a few studies on antibody production are reported in microalgae, it is considered that this topic offers great opportunities to express those antibodies that have already been shown functional on the treatment of relevant diseases or useful in diagnostics.

Protein as biopharmaceuticals

In a remarkable contribution, Rasala et al. (2010) assessed the expression of seven biopharmaceuticals in chloroplasts comprising erythropoietin, 10FN3, 14FN3, interferon-β, proinsulin, VEGF and HMGB1, proteins that represent a wide diverse group. To become a viable protein production platform, algal chloroplasts must not only express recombinant proteins but those proteins must also be biologically active in a highly purified state. Importantly, proteins 14FN3, VEGF and HMGB1 were successfully expressed in soluble form and affinity purified using FLAG affinity chromatography to the C-terminal FLAG epitope.

Algal-derived VEGF retained its antigenic properties as revealed by an ELISA assay, suggesting a proper folding. After determining the concentration of VEGF using *E. coli*-derived VEGF as standard, receptor binding assay reflected that the algal-derived VEGF has a dose-dependent binding to the VEGF receptor with a slightly lower affinity in comparison to the bacterial-derived protein. Authors hypothesized that this phenomenon might be due to fractions of misfolded or truncated VEGF present in the assayed samples. After performing the VEGF-receptor binding competition assay it was observed that *E. coli*-derived VEGF competed with algae-derived VEGF for VEGF-receptor binding. In accordance with the previous results from affinity assay, the *E. coli*-derived VEGF displaced the algal-derived VEGF (200 ng/mL) from VEGFR with an IC50 of approximately 40 ng/mL.

The functionality of the algal-derived HMGB1 was demonstrated by an external group (Bio-Quant, San Diego, CA, USA) using a fibroblast chemotaxis assays, observing that it compared with the *E. coli*-derived HMGB1. In summary, this report demonstrated that *C. reinhardtii* chloroplasts are capable of expressing bioactive VEGF and HMGB1 (Table 3). The functionality assay for the 14FN3 protein remains to be developed.

EPO, 10FN3, interferon-β and proinsulin proteins were not expressed at significant levels, probably due to stability

problems, and therefore further strategies to achieve the efficient expression of these proteins remain to be elucidated.

Other proteins and secondary metabolites of biotechnological relevance in *C. reinhardtii*

Because *C. reinhardtii* is an edible alga, production of therapeutic proteins in this organism offers the potential for oral delivery of gut-active proteins, such as M-SAA (mammalian coding region of bovine mammary-associated serum amyloid), which stimulates the production of mucin in the gut, acting in the prophylaxis of bacterial and viral infections. Manuell et al. (2007) transformed *C. reinhardtii* chloroplast with the chimeric *psbA-m-saa* gene which results in the accumulation of recombinant M-SAA above 5% of total protein; this protein could be a protective agent for mammals that lack a source of colostrum. These results demonstrate that *C. reinhardtii* can be used as platform for biologically active mammalian therapeutic proteins.

Recently, another area has been explored by Yoon et al. (2011) using transgenic microalgae as a food additive to deliver dietary enzymes without the need for protein purification engineering reported. They introduced the *E. coli* appA phytase gene (generate Chlasate activity) into the chloroplast of *C. reinhardtii*, and concluded that this recombinant Chlasate reduced the fecal phytate excretion when used to feed young broiler chicks at 500 U/kg of body weight. An increase of 2% on the inorganic phosphate was also observed in comparison to those fed with the basal diet. These results showed that *C. reinhardtii* can be used as platform of both the food and pharmaceutical industries.

Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich heavy metal binding proteins with multifunction, such as metal detoxification and antioxidation. Zheng et al. (2006) assayed the expression of human metallothionein-2 (MT2) in chloroplast of *C. reinhardtii*; additionally, transgenic clones demonstrated a higher resistance than wild-type to UV-B exposure under all the examined conditions. This approach can be exploited to produce high quantities of this protein to obtain highly purified recombinant human MT2 protein with native characteristics at low cost in order to apply it as additive in cosmetics protecting against oxidative damage and UV radiation. Alternatively, it was previously demonstrated that the heavy metal binding properties of *C. reinhardtii* can be enhanced by introducing the foreign metallothionein-like (MT-like) gene into the nuclear genome (Cai et al. 1999; Hu et al. 2002; Han et al. 2008) demonstrating the advantage of this system in other areas such as bioremediation.

In addition to vaccines, antibodies and pharmaceuticals, other valuable compounds as such as secondary metabolites have been produced in this model by means of engineering the expression of the genes responsible for their biosynthesis. In particular, the geranylgeranyl pyrophosphate synthase is a crucial enzyme for the terpenoid biosynthesis that has been expressed in *C. reinhardtii*. The chloroplast-encoded enzyme was successfully expressed using a gene derived from the thermophilic Archae *Sulfolobus acidocaldarius*. (Fukasaki et al. 2003). A modest enzyme accumulation of 0.1% of the TSP is reported, probably due to the lack of using a codon-optimized gene, which might increase protein accumulation up to 80-fold in the chloroplast (Franklin et al. 2002).

Furthermore, carotenoids have been produced at higher yields by means of genetic chloroplast manipulation of *C. reinhardtii*, using a gene from *Haematococcus pluvialis*, encoding a β-carotene hydrolase (Tan et al. 2007) or a phytoene synthase gene isolated from *Chorella zofingiensis.* A significant enhancement in violaxanthin and lutein content was achieved, reaching a 2- and 2.2-fold increase in comparison to the untransformed control cells, respectively (Cordero et al. 2011).

Future challenges on process engineering aspects

C. reinhardtii offers many advantages as a recombinant protein expression system as aforementioned; however, a major concern is the high production of the recombinant protein, since some therapeutic proteins, like antibodies, are required in huge amounts, then the availability of bioproduct needed for a specific application can be limited. It is known that *C. reinhardtii* can be cultivated in large scale in open ponds, but also, cultivation could be carried out in photobioreactors (PBRs).

PBRs have more advantages than open ponds because they are close systems and the most important growth parameters as light intensity (2,500–5,000 lux), temperature (18–24°C), pH (8.2–8.7), nutrient quantity, carbon dioxide (1.85 g CO_2/g biomass or higher) can be monitored and controlled, getting a robust bioprocess. One of the major limitations for any large-scale algal culture is the highly non-homogeneous light distribution within the photobioreactor, since biomass concentration is many orders of magnitude higher and the strong optical density of pigments limits light penetration, resulting in an overall reduced photosynthetic yield and consequently a lower productivity as was reported for *Dunaliella salina* (Neidhardt et al. 1998; Melis et al. 1998, 2009). Furthermore, Janssen et al. (2000) designed an air-lift loop bioreactor for cultivation of microalgae (*C. reinhardtii* and *Dunaliella tertiolecta*), reporting that efficient mixing, combined with a controlled liquid flow, a carbon dioxide supply and oxygen removal do not limit the productivity of this

reactor. Maximal productivity was reached when continued illumination at 333–389 Wm^{-2}, was provided. These results denote the importance of the relation between medium, photoperiod and biomass yield. Additionally cultivating the microalgae in PBRs with a high surface/volume ratio is recommended for the design of a PBR with a maximum productivity, which maintains a relatively high specific light supply rate (Zijffers et al. 2010).

An alternative strategy to phototropic cultivation is the heterotrophic approach that can be well controlled and provides the possibility of achieving fast growth and high yields of valuable products on a large scale for some algae species (Chen and Chen 2006).

Additionally, a major challenge is the development of methodologies for large-scale recombinant protein production in *C. reinhardtii* in order to facilitate the progress at industrial level. These scale-up methodologies should include the following operational parameters: height/diameter ratio of the reactor, flow velocity, transfer mass coefficient (kla), mixing rate, initial density and light levels, in conjunction with nutrient concentration, pH, salinity and temperature; all these factors may affect the recombinant proteins production in microalgae.

On the other hand, only few studies have been carried out for process optimization in *C. reinhardtii*. For example, some research groups have modified nutrient concentration or operational variables such as cell concentration, light intensity and pH to optimize hydrogen production (Kosourov et al. 2002; Hahn et al. 2004; Jo et al. 2006). Jo et al. (2006) concluded that the response surface methodology (RSM) successfully located optimal conditions that maximized both the algal growth and hydrogen production rates with respect to the effects of pH and nutrient concentrations in TAP medium. In fact, recombinant protein optimization in *C. reinhardtii* has not been explored; thus, further work is necessary to address large-scale production and optimization, in order to establish a platform for commercialization of recombinant proteins produced in *C. reinhardtii*. In addition to RSM, it is proposed to use orthogonal designs when several parameters are assayed in the process optimization. As an alternative to RSM, the Taguchi method that is a simple statistical tool involving a system of tabulated arrays that allows a maximum number of major effects to be estimated in an orthogonal fashion with a minimum number of experimental runs; this method can also be applied to predict the significant contribution of the design variable(s) and the optimum combination of each variable for elucidating the optimal yield of the process by conducting experiments on a real-time basis. Then it will be interesting to examine this methodology with specific case studies in the field of biotechnology, particularly in recombinant proteins produced in *C. reinhardtii*.

Conclusions and perspectives

Different expression systems have successfully been employed to produce a large variety of proteins, including biopharmaceuticals and enzymes with implication on the production of health-promoting compounds. It is essential, however, to determine which system offers most advantages for the production of the protein of interest and the route of delivery. The ideal expression system for a particular case would be that producing the safest, biologically active material at the lowest cost and convenient production yields. The use of mammalian cells to produce recombinant proteins has the advantage that these proteins are identical to those of human origin; however, the culture of these cells is expensive and can only be carried out on a limited scale. Although bacteria can be used to produce recombinant proteins on a large scale, they have the disadvantage of lacking many of the post-translational modifications required for optimal biological activity of complex proteins. As eukaryotic organisms, transgenic plants overcome this obstacle and have been widely demonstrated that plants offer an economic system for expression of certain proteins in the biotechnology industry (Hiatt et al. 1988; Daniell et al. 2001; Peters and Stoger 2011). However, there are some inherent weaknesses using this approach, probably the most significant one is the length of time required from the initial transformation event to obtain usable (mg to g) quantities of recombinant protein. It has been estimated that it can take more than 1 year for crops such as tobacco, and over 3 years for species such as corn. Another important concern is related to the potential of gene flow (via pollen) to surrounding crops when typical nuclear transformation is employed (Ellstrand 2001), although the use of culture plant cell in bioreactors can also be accomplished. Consequently, from both a timescale and a regulatory perspective, transgenic microalgae might be the best model for the expression of biopharmaceuticals.

Some years ago, *C. reinhardtii* emerged as a notable candidate for the expression of recombinant proteins specified by the inherent biology of the organism, the easiness with which it can be cultured (they have the ability to grow phototrophically or heterotrophically using acetate as carbon source), the well-established nuclear and chloroplast transformation procedures and the wide scale on which it can be grown. These advantages allow to develop transformed lines in a short period of time and protein production can be evaluated at flask scale in as little as 6 weeks, with the potential to scale up in another 4–6 weeks (Mayfield and Franklin 2005).

However, there are certainly obstacles to be overcome before the full potential of recombinant protein expression in *C. reinhardtii* can be exploited. As other systems,

the attainable protein expression levels are crucial to many applications in molecular farming. In addition to codon optimization, several other avenues have been examined to increase recombinant protein expression in the chloroplast, and much of this work has benefited from the current state of knowledge concerning endogenous gene expression in the chloroplast. The complex interplay of many other factors, including enhancer elements, regulatory mechanisms, transformation-associated events, sensitivity to proteases and protein localization, underlines and underscores the difficulties in establishing a standard system for recombinant protein production in microalgae. However, transgene expression can also been influenced by several factors that cannot be controlled precisely through construct design, which leads to variable transgene expression and, in some cases, its complete inactivation. Such factors include the position of transgene integration, the structure of the transgenic locus, gene-copy number and the presence of truncated or rearranged transgene copies.

Once transgenic strains are obtained, the cultivation operating parameters, including agitation, illumination, growth media composition, temperature, pH and CO_2 concentration, can also significantly affect protein yields. The factors discussed in the present review deal with the "upstream" side of process engineering and design. Cooperation among researchers on upstream bioprocess design will further facilitate the development of economically viable transgenic microalgae-based bioreactors. Access to several alternative approaches to optimize protein yields augurs well for the safe and economical production of biopharmaceuticals in microalgae.

References

Adam Z, Rudella A, van Wijk KJ (2006) Recent advances in the study of *CLp*, *FtsH* and other protease located in chloroplast. Curr Opin Plant Biol 9:234–240

Alexandersen S, Mowat GN (2005) Foot-and-mouth disease: host range and pathogenesis. Curr Top Microbiol Immunol 288:9–42

Artsaenko O, Peisker M, zur Nieden U, Fiedler U, Weiler EW, Müntz K, Conrad U (1995) Expression of a single-chain Fv antibody against abscisic acid creates a wilt phenotype in transgenic tobacco. Plant J 8:745–750

Bach JF, Chatenoud L (2001) Tolerance to islet autoantigens in type 1 diabetes. Annu Rev Immunol 19:131

Barnes D, Franklin S, Schultz J, Henry R, Brown E, Coragliotti A, Mayfield SP (2005) Contribution of 5′ and 3′ untranslated regions of plastid mRNAs to the expression of *Chlamydomonas reinhardtii* chloroplast genes. Mol Genet Genomics 274:625–636

Baum TJ, Hiatt A, Parrott WA, Pratt LH, Hussey RS (1996) Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode. Mol Plant Microbe Interact 9:382–387

Bentley DL (2005) Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol 17:251–256

Berthold P, Schmitt R, Mages W (2002) An engineered *Streptomyces hygroscopicus aph 7*-gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. Protist 153:401–412

Blankenship JE, Kindle KL (1992) Expression of chimeric genes by the light-regulatable cabll-1 promoter in *Chlamydomonas reinhardtii*. A *cabll*-1/*nit*l gene functions as a dominant selectable marker in a nit1^{-} nit2^{-} strain. Mol Cell Biol 12:5268–5279

Blowers AD, Klein U, Ellmore GS, Bogorad L (1993) Functional in vivo analyses of the 3′ flanking sequences of the *Chlamydomonas* chloroplast *rbcL* and *psaB* genes. Mol Gen Genet 238:339–349

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312:425–438

Boehm R (2007) Bioproduction of therapeutic proteins in the 21st century and the role of plants and plant cells as production platforms. Ann NY Acad Sci 1102:121–134

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson B, Robertson D, Klein T, Shark K (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534–1538

Britt AB, May GD (2003) Re-engineering plant gene targeting. Trends Plant Sci 8:90–95

Buikema WJ, Haselkorn R (2001) Expression of the Anabaena *hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc Natl Acad Sci USA 98:2729–2734

Cai XH, Brown C, Adhiya J, Traina SJ, Sayre RT (1999) Growth and heavy metal binding properties of transgenic *Chlamydomonas* expressing a foreign metallothionein gene. Int J Phytoremediat 1:53–65

Campbell WH, Gowri G (1990) Codon usage in higher plants, green algae, and cyanobacteria. Plant Physiol 92:1–11

Cerutti H, Johnson AM, Gillham NW, Boynton JE (1997) A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. Genetics 145:97–110

Chen GQ, Chen F (2006) Growing phototrophic cells without light. Biotechnol Lett 28:607–616

Conrad U, Fiedler U (1998) Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol Biol 38:101–109

Coragliotti AT, Beligni MV, Franklin SE, Mayfield SP (2011) Molecular factors affecting the accumulation of recombinant proteins in the *Chlamydomonas reinhardtii* chloroplast. Mol Biotechnol 48:60–75

Cordero BF, Couso I, León R, Rodríguez H, Vargas MA (2011) Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoenosynthase gene isolated from *Chlorella zofingiensis*. Appl Microbiol Biotechnol 91:341–351

Croft MT, Moulin M, Webb ME, Smith AG (2007) Thiamine biosynthesis in algae is regulated by riboswitches. Proc Natl Acad Sci USA 104:20770–20775

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219–226

Dauvillée D, Delhaye S, Gruyer S, Slomianny C, Moretz SE, D'Hulst C, Long CA, Ball SG, Tomavo S (2010) Engineering the chloroplast targeted malarial vaccine antigens in *Chlamydomonas* starch granules. PLoS One 5:e15424

De Neve M, De Loose M, Jacobs A, Van Houdt H, Kaluza B, Weidle U, Van Montagu M, Depicker A (1993) Assembly of an antibody and its derived antibody fragment in *Nicotiana* and *Arabidopsis*. Transgenic Res 2:227–237

Dreesen IA, Charpin-El Hamri G, Fussenegger M (2010) Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. J Biotechnol 145:273–280

Dubovsky F, Rabinovich NR (2004) Malaria vaccines. In: Plotkin SA, Orenstein WA (eds) vaccines, 4th edn. Philadelphia, Saunders, pp 1283–1289

Eberhard S, Drapier D, Wollman FA (2002) Searching limiting steps in the expression of chloroplast-encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*. Plant J 31:149–160

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop HU (1999) In vivo analysis of plastid *psbA*, *rbcL* and *rpl32* UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J 19:333–345

Eichler-Stahlberg A, Weisheit W, Ruecker O, Heitzer M (2009) Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. Planta 229:873–883

Ellstrand NC (2001) When transgenes wander, should we worry? Plant Physiol 125:1543–1545

Ferrante P, Catalanotti C, Bonente G, Giuliano G (2008) An optimized, chemically regulated gene expression system for *Chlamydomonas*. PLoS One 3:e3200

Fischer N, Rochaix JD (2001) The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*. Mol Genet Genomics 265:888–894

Food Drug Adm (FDA) (2002) Guidance for industry: drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. http://www.fda.gov/cber/gdlns/bioplant.htm

Foster TJ, Hook M (1998) Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 6:484–488

Franklin SE, Mayfield SP (2004) Prospect for molecular farming in the green alga *Chlamydomonas reinhardtii*. Curr Opin Plant Biol 7:159–165

Franklin S, Mayfield SP (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin Biol Ther 5:225–235

Franklin S, Ngo B, Efuet E, Mayfield SP (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. Plant J 30:733–744

Fukasaki EI, Nishikawa T, Kato K, Shinmyo A, Hemmi H, Nishino T, Kobayashi A (2003) Introduction of the archaebacterial geranylgenanyl pyrophosphate synthase gene into *Chlamydomonas reinhardtii* chloroplast. J Biosci Bioeng 95:283–287

Gallie DR (1998) A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Gene 216:1–11

Goldenberg D, Azar I, Oppenheim AB, Brandi A, Pon CL, Gualerzi CO (1997) Role of *Escherichia coli csp*A promoter sequences and adaptation of translational apparatus in the cold shock response. Mol Gen Genet 256:282–290

Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. Nucleic Acids Res 19:4083–4089

Granell A, Fernández del-Carmen A, Orzáez D (2010) In planta production of plant-derived and non-plant-derived adjuvants. Expert Rev Vaccines 9:843–858

Grubman MJ, Baxt B (2004) Foot-and-mouth disease. Clin Microbiol Rev 17:465–493

Hahn JJ, Ghirardi ML, Jacoby WA (2004) Effect of process variables on photosynthetic algal hydrogen production. Biotechnol Prog 20:989–991

Han SH, Hu ZL, Lei AP (2008) Expression and function analysis of the metallothionein-like (MT-like) gene from *Festuca rubra* in *Chlamydomonas reinhardtii* chloroplast. Sci China Ser C-Life Sci 51:1076–1081

Harris EH (1989) The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use. Academic Press, Inc, New York

He DM, Qian KX, Shen GF, Zhang ZF, Li YN, Su ZL, Shao HB (2007) Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in *Chlamydomonas reinhardtii* chloroplasts. Colloids Surf B Biointerfaces 55:26–30

Hefferon KL, Xu J, AbouHaidar MG (2000) Identification and in vivo expression of a prokaryotic-like ribosome recognition sequence upstream of the coat protein gene of potato virus X. Arch Virol 145:945–956

Heitzer M, Eckert A, Fuhrmann M, Griesbeck C (2007) Influence of codon bias on the expression of foreign genes in microalgae. Adv Exp Med Biol 616:46–53

Herz S, Fussl M, Steiger S, Koop HU (2005) Development of novel types of plastid transformation vectors and evaluation of factors controlling expression. Trans Res 14:969–982

Hiatt A, Cafferky R, Bowdish K (1988) Production of antibodies in transgenic plants. Nature 342:76–78

Hu ZL, Xing M, Wu YH, Tu H (2002) The comparison of heavy metal binding properties and tolerance between transgenic strains and wild type in *Chlamydomonas reinhardtii*. J Lake Sci 14:247–252

Hunt AG, Maiti IB (2001) Strategies for expressing multiple foreign genes in plants as polycistronic constructs. In vitro Cell Dev Biol Plant 37:313–320

Janssen M, de Bresser L, Baijens T, Tramper J, Mur LR, Snel JFH, Wijffels RH (2000) Scale-up aspects of photobioreactors: effects of mixing-induced light/dark cycles. J App Phycol 12:225–237

Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. New Phytol 179:257–285

Jo JH, Lee DS, Park JM (2006) Modeling and optimization of photosynthetic hydrogen gas production by green alga *Chlamydomonas reinhardtii* in sulfur-deprived circumstance. Biotechnol Prog 22:431–437

Kasai S, Yoshimura S, Ishikura K, Takaoka Y, Kobayashi K, Kato K, Shinmyo A (2003) Effect of coding regions on chloroplast gene expression in *Chlamydomonas reinhardtii*. J Biosci Bioeng 95:276–282

Kato K, Marui T, Kasai S, Shinmyo A (2007) Artificial control of transgene expression in *Chlamydomonas reinhardtii* chloroplast using the lac regulation system from *Escherichia coli*. J Biosci Bioeng 104:207–213

Kindle KL (1998) Nuclear transformation: technology and applications. In: Rochaix JD, Goldschmidt-Clermont M, Merchant S (eds) The Molecular biology of chloroplast and mitochondria in *Chlamydomonas*. Kluwer Academic Publishers Dordrecht, The Netherlands, pp 41–61

Kluytmans J, van Belkum A, Verbrugh H (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 10:505–520

Kosourov S, Tsygankov A, Seibert M, Ghirardi ML (2002) Sustained hydrogen photoproduction by *Chlamydomonas reinhartii*: effects of culture parameters. Biotechnol Bioeng 78:731–740

Koziel MG, Carozzi NB, Desai N (1996) Optimizing expression of transgenes with an emphasis on post-transcriptional events. Plant Mol Biol 32:393–405

Kozminski KG, Diener DR, Rosnbaum JL (1993) High level expression of non-acetylatable alpha tubulin in *Chlamydomonas reinhardtii*. Cell Motil Cytosketelon 25:158–170

Kucho K, Ohyama K, Fukuzawa H (1999) CO_2-responsive transcriptional regulation of CAH1 encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. Plant Physiol 121:1329–1338

Lareau LF, Green RE, Bhatnagar RS, Brenner SE (2004) The evolving roles of alternative splicing. Curr Opin Struct Biol 14:273–282

Li SS, Tsai HJ (2009) Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. Fish Shellfish Immunol 26:316–325

Liu M, Grigoriev A (2004) Protein domains correlate strongly with exons in multiple eukaryotic genomes-evidence of exon shuffling? Trends Genet 20:399–403

Liu K, Sandgren EP, Palmiter RD, Stein A (1995) Rat growth hormone gene introns stimulate nucleosome alignment in vitro and in transgenic mice. Proc Natl Acad Sci USA 92:7724–7728

Lumbreras V, Stevens DR, Purton S (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. Plant J 14:441–447

Ma JC, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nature Rev 4:794–805

Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, Weber A, McDonald TL, Mayfield SP (2007) Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. Plant Biotechnol J 5:402–412

Mayfield SP, Franklin SE (2005) Expression of human antibodies in eukaryotic micro-algae. Vaccine 23:1828–1832

Mayfield SP, Schultz J (2004) Development of a luciferase reporter gene, *luxCt*, for *Chlamydomonas reinhardtii* chloroplast. Plant J 37:449–458

Mayfield SP, Franklin SE, Lerner RA (2003) Expression and assembly of a fully active antibody in algae. Proc Natl Acad Sci USA 100:438–442

Mayfield SP, Manuell AL, Chen S, Wu J, Tran M, Siefker D, Muto M, Marin-Navarro J (2007) *Chlamydomonas reinhardtii* chloroplasts as protein factories. Curr Opin Biotechnol 18:126–133

Melis A (2009) Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. Plant Sci 177:272–280

Melis A, Neidhardt J, Benemann JR (1998) *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. J Appl Phycol 10:515–525

Mellies J, Brems R, Villarejo M (1994) The *Escherichia coli pro U* promoter element and its contribution to osmotically signaled transcription activation. J Bacteriol 176:3638–3645

Muto M, Henry RE, Mayfield SP (2009) Accumulation and processing of a recombinant protein designed as a cleavable fusion to the endogenous *Rubisco LSU* protein in *Chlamydomonas* chloroplast. BMC Biotechnol 9:26

Nakamura Y, Gojobori T, Ikemura T (1999) Codon usage tabulated from the international DNA sequence databases; its status 1999. Nucleic Acids Res 27:292–298

Neidhardt J, Benemann JR, Zhang L, Melis A (1998) Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae). Photosynth Res 56:175–184

Nelson JAE, Savereide PB, Lefebvre PA (1994) The CRY1 gene in *Chlamydomonas reinhardtii*: structure and use as a dominant selectable marker for nuclear transformation. Mol Cell Biol 14:4011–4019

Neupert J, Karcher D, Bock R (2009) Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. Plant J 57:1140–1150

Newman SM, Boynton JE, Gillham NW, Randolph-Anderson BL, Johnson AM, Harris EH (1990) Transformation of chloroplast ribosomal RNA in *Chlamydomonas*: molecular and genetic characterization of integration events. Genetics 126:875–888

Nickelsen J (2003) Chloroplast RNA-binding proteins. Curr Genet 43:392–399

Ohresser M, Matagne RF, Loppes R (1997) Expression of the arylsulphatase reporter gene under the control of the nit1 promoter in *Chlamydomonas reinhardtii*. Curr Genet 31:264–271

Passwater R, Soloman N (eds) (1997) Algae: the next generation of superfoods. Exp Op Health J 1:2–10

Peacock SJ, de Silva l, Lowy FD (2001) What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol 9:605–610

Peters J, Stoger E (2011) Transgenic crops for the production of recombinant vaccines and anti-microbial antibodies. Hum Vaccin 1:7

Potvin G, Zhang Z (2010) Strategies for high-level recombinant protein expression in transgenic microalgae: a review. Biotechnol Adv 28:910–918

Preiss S, Schrader S, Johanningmeier U (2001) Rapid, ATP-dependent degradation of a truncated D1 protein in the chloroplast. Eur J Biochem 268:4562–4569

Puigbo P, Guzmán E, Romeu A, Garcia-Vallve S (2007) OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res 35:W126–W131

Puigbo P, Bravo IG, Garcia-Vallve S (2008) E-CAI: a novel server to estimate an expected value of Codon Adaptation Index (eCAI). BMC Bioinformatics 9:65

Quinn JM, Merchant S (1995) Two copper-responsive elements associated with the *Chlamydomonas Cyc6* gene function as target for transcriptional activator. Plant Cell 7:623–638

Quinn JM, Kropat J, Merchant S (2003) Copper response element and Crr1-dependent Ni2-responsive promoter for induced, reversible gene expression in *Chlamydomonas reinhardtii*. Eukaryot Cell 2:995–1002

Rasala BA, Muto M, Lee PA, Jager M, Cardoso RM, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnol J 8:719–733

Rasala BA, Muto M, Sullivan J, Mayfield SP (2011) Improved heterologous protein expression in the chloroplast of *Chlamydomonas reinhardtii* through promoter and 5′ untranslated region optimization. Plant Biotechnol J 9:674–683

Riehle MM, Markianos K, Niare O, Xu J, Li J, Toure AM, Podiougou B, Oduol F, Diawara S, Diallo M, Coulibaly B, Ouatara A (2006) Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. Science 312:577–579

Rochaix JD (1996) Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas*. Plant Mol Biol 32:327–341

Rochaix J, Fischer N, Hippler M (2000) Chloroplast site-directed mutagenesis of photosystem I in *Chlamydomonas*: electron transfer reactions and light sensitivity. Biochimie 82:635–645

Rott R, Liveanu V, Drager RG, Stern DB, Schuster G (1998) The sequence and structure of the 3′-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. Plant Mol Biol 36:307–314

Salvador ML, Suay L, Anthonisen IL, Klein U (2004) Changes in the 5′-untranslated region of the *rbcL* gene accelerates transcript degradation more than 50-fold in the chloroplast of *Chlamydomonas reinhardtii*. Curr Genet 45:176–182

Salyaev RK, Rigano MM, Rekoslavskaya NI (2010) Development of plant-based mucosal vaccines against widespread infectious diseases. Expert Rev Vaccines 9:937–946

Schroda M (2006) RNA silencing in *Chlamydomonas*: mechanisms and tools. Curr Genet 49:69–84

Schroda M, Blocker D, Beck CF (2000) The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. Plant J 21:121–131

Searle PFG, Stuart W, Palmiter RD (1985) Building a metal-responsive promoter with synthetic regulatory elements. Mol Cell Biol 5:1480–1489

Serfling E, Lubbe A, Dorsch-Hasler K, Schaffner W (1985) Metal-dependent SV40 viruses containing inducible enhancers from the upstream region of metallothionein genes. EMBO J 4:3851–3859

Shimizu Y (1996) Microalgal metabolites: a new perspective. Annu Rev Microbiol 50:442–445

Soria-Guerra RE, Moreno-Fierros L, Rosales-Mendoza S (2011) Two decades of plant-based candidate vaccines: a review of the chimeric protein approaches. Plant Cell Rep 30:1367–1382

Stern DB, Radwanski ER, Kindle KL (1991) A 3′ stem/loop structure of the *Chlamydomonas* chloroplast *atpB* gene regulates mRNA accumulation in vivo. Plant Cell 3:285–297

Stevens DR, Rochaix JD, Purton S (1996) The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. Mol Gen Genet 251:23–30

Su ZL, Qian KX, Tan CP, Meng CX, Qin S (2005) Recombination and heterologous expression of allophycocyanin gene in the chloroplast of *Chlamydomonas reinhardtii*. Acta Biochim Biophys Sin (Shanghai) 37:709–712

Suay L, Salvador ML, Abesha E, Klein U (2005) Specific roles of 5′ RNA secondary structures in stabilizing transcripts in chloroplasts. Nucleic Acids Res 33:4754–4761

Subbarao SK, Sharma VP (1997) Anopheline species complexes and malaria control. Indian J Med Res 106:164–173

Sun M, Qian K, Su N, Chang H, Liu J, Shen G (2003) Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. Biotechnol Lett 25:1087–1092

Surzycki R, Cournac L, Peltier G, Rochaix JD (2007) Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. Proc Natl Acad Sci U S A 104:17548–17553

Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, Rochaix JD, Ajam T, Surzycki S (2009) Factors effecting expression of vaccines in microalgae. Biologicals 37:133–138

Tan CP, Zhao FQ, Su ZL, Liang CW, Qin S (2007) Expression of β-carotene hydroxylase gene (crtR-B) from the green alga *Haematococcus pluvialis* in chloroplast of *Chlamydomonas reinhartii*. J Appl Phycol 19:347–355

Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP (2009) Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. Biotechnol Bioeng 104:663–673

Vaistij FE, Goldschmidt-Clermont M, Wostrikoff K, Rochaix JD (2000) Stability determinants in the chloroplast *psbB/T/H* mRNAs of *Chlamydomonas reinhardtii*. Plant J 21:469–482

Van der Vyver C, Schneidereit J, Driscoll S, Turner J, Kunert K, Foyer CH (2003) Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. Plant Biotechnol J 1:101–112

Voigt J, Fausel M, Bohley P, Adam K, Marquardt O (2004) Structure and expression of the ornithine decarboxylase gene of *Chlamydomonas reinhardtii*. Microbiol Res 159:403–417

Wang XF, Brandsma M, Tremblay R, Maxwell D, Jevnikar AM, Huner N, Ma S (2008) A novel expression platform for the production of diabetes-associated autoantigen human glutamic acid decarboxylase (hGAD65). BMC Biotechnol 8:e87

Weathers PJ, Towler MJ, Xu J (2010) Bench to batch: advances in plant cell culture for producing useful products. Appl Microbiol Biotechnol 85:1339–1351

Wells SE, Hillner PE, Vale RD, Sachs AB (1998) Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell 2:135–140

WHO (2005) World Malaria Report. World Health Organization

Wirth S, Calamante G, Mentaberry A, Bussmann L, Lattanzi M, Baranao L, Bravo-Almonacid F (2004) Expression of active human epidermal growth factor (*hEGF*) in tobacco plants by integrative and non-integrative systems. Mol Breed 13:23–35

Xia X (2007) An improved implementation of codon adaptation index. Evol Bioinformatics 3:53–58

Yoon SM, Kim SY, Li KF, Yoon BH, Choe S, Kuo MMC (2011) Transgenic microalgae expressing *Escherichia coli AppA* phytase as feed additive to reduce phytate excretion in the manure of young broiler chicks. Appl Microbiol Biotechnol 91:553–563

Zheng YK, Shen GF, Ru BG (2006) Survival of human metallothionein-2 transplastomic *Chlamydomonas reinhardtii* to ultraviolet B exposure. Acta Biochim Biophys sin 38:187–193

Zijffers JWF, Schippers KJ, Zheng K, Janssen M, Tramper J, Wijffels RH (2010) Maximum photosynthetic yield of green microalgae in photobioreactors. Mar Biotechnol 12:708–718