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Research review paper

Strategies for high-level recombinant protein expression in transgenic microalgae: A review

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ABSTRACT

Microalgae represent the 'best of both worlds', combining the high growth rate and ease of cultivation of microorganisms with the ability to perform post-transcriptional and post-translational modifications of plants. The development of economically viable microalgal expression systems is, however, hindered by low recombinant protein yields. Although there are still many obstacles to overcome before microalgae become standard expression systems, considerable progress has been made in recent years in regards to elucidating the causes for these low yields and in the development of strategies to improve them. Transgenes have successfully been expressed in both nuclear and chloroplast microalgal genomes, although at economically viable levels only in the latter. The present review describes recent progress in genetic manipulation of microalgae, outlines strategies to increase protein yields and presents some interesting avenues of research that remain to be explored.

Contents

1. Introduction

Harnessing the benefits of microalgae is by no means a novel concept. Historical records indicate that Chinese civilizations used *Nostoc*, *Arthrospira* and *Aphanizomenon* blue-green algae as alternative food sources to stave off famine as early as 2000 years ago, a strategy later employed by Aztec civilizations in the 14th–16th centuries. The large-scale cultivation of microalgae, however, only began shortly after WWII when the United States, Japan and Germany were facing dwindling food reserves and were forced to consider alternate sources of protein for booming populations. The current

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world production of raw microalgal biomass exceeds 5000 tonnes, generating an estimated 1–1.25 billion USD in revenue every year (Pulz and Gross, 2004; Walker et al., 2005a; Spolaore et al., 2006).

Today microalgae are still mainly used as nutritional supplements for human and animal consumption due to their high protein and vitamin contents. They have, however, also elicited interest as production systems for a host of valuable naturally-produced compounds (Harun et al., 2010). The most notable of these applications is the cultivation of the green algae *Haematococcus pluvialis* for its high-level (50 mg/g DCW) production of astaxanthin, a carotenoid pigment widely used as a feed additive in fish aquaculture, and prized by the pharmaceutical and cosmetic industries for its antioxidant properties (Hyunsuk et al., 2005; Kathiresan and Sarada, 2009).

Given their relatively high oil content, microalgae are of considerable interest for the biodiesel industry (Williams and Laurens, 2010). Although microalgal oil contents as high as 75% DCW have been reported, such contents are highly unusual and obtained only under of specific cultivation conditions, and therefore do not apply to current industrial systems. The microalgal systems used in practical applications typically have oil contents closer to 20–40% DCW. It has been argued that microalgae may present the only economically and environmentally viable feedstock for biofuel production. Estimates based on oil contents of 70% DCW, which some believe to eventually be reachable as research progresses, place the cost of microalgae-derived biodiesel at 0.72 USD/L, which currently exceeds the viability of alternate biofuel feedstocks (Chisti, 2007). Considerable progress has been achieved in regards to increasing the oil yield of microalgae (Courchesne et al., 2009), and several reviews on microalgae-based biodiesel production have recently been published (Gressel, 2008; Li et al., 2008; Meng et al., 2009; Sialve et al., 2009; Brennan and Owende, 2010; Greenwell et al., 2010; Mata et al., 2010; Smith and Sturm, 2010; Williams and Laurens, 2010). Microalgae cultivation methods are reviewed by Carvalho et al. (2006); Eriksen (2008) and Xu et al. (2009).

In addition to their value as platforms for the production of naturally-produced compounds, transgenic microalgae have been eliciting considerable interest over the last decade as recombinant protein expression systems, as they combine the rapid growth and ease of cultivation inherent to many microorganisms with the ability of plant cells to perform post-transcriptional and post-translational modifications. Research into transgenic microalgae is fuelled by the worldwide demand for recombinant proteins and other bioproducts, the market for which is growing exponentially and is expected to reach USD 70 billion by the end of 2010 (Pavlou and Reichert, 2004).

2. Current state of bioreactor technology

Bacterial and yeast-based bioreactors are the most widely used systems for the production of recombinant proteins, as they are very well characterized, their genomes easy to manipulate, and their cultivation simple and inexpensive (Swartz, 2001). Bacteria, however, do not perform the post-transcriptional and post-translational modifications, including glycosylation, phosphorylation and disulfide bond formation, required for the correct folding and assembly of more complex proteins. Although eukaryotic yeasts can perform these modifications, their profiles are unsuitable for therapeutic proteins destined for animal or human consumption. Recombinant proteins in yeast are usually hyperglycosylated, which alters immunogenic epitopes, and the high-mannose glycosylation performed in such systems results in low *in vivo* half-life of proteins, both factors which compromise the therapeutic activity of the products (Wildt and Gerngross, 2005).

To overcome these difficulties, mammalian, insect, or plant cell bioreactors are used. Several recombinant eukaryotic proteins have been correctly synthesized, processed, and harvested in these cell-based reactors, and have been approved for use in therapeutic applications (Wurm, 2004; Boehm, 2007; Weathers et al., 2010). Mammalian cell-based bioreactors are, however, very expensive to develop and maintain, and are plagued by complex nutrient requirements, poor oxygen and nutrient distribution, waste accumulation, contamination by pathogens, and high sensitivity of cells to shear stress (Wurm, 2004; Zhang et al., 2010). Compared to mammalian cells, insect cells are easier to culture, are more tolerant to osmolality changes and by-product accumulation, and baculovirus-infected insect cells generally lead to higher recombinant protein expression levels. They do, however, also have complex nutrient requirements, and baculovirus-infection systems, due to their lytic operating mechanism, lead to the release of endogenous proteases that significantly lower product yields (Ikonomou et al., 2003).

Plant-based reactors, although much less expensive than their mammalian and insect counterparts and resistant to most animal-infecting pathogens, have slow growth cycles and are linked with concerns relating to environmental contamination by genetically modified plants. Although differences are observed in glycosylation profiles between animal and plant cells, in many cases the stability, correct folding and resistance to proteases of mammalian-derived products have not been significantly affected. Plant-derived proteoglycans have, however, been linked to allergic reactions, which is a major concern for recombinant proteins destined for human therapeutic applications (Decker and Reski, 2004).

Although transgenic microalgal technology is still in its infancy, microalgae may represent the 'best of both worlds' by combining simple and inexpensive growth requirements and capabilities for post-transcriptional and -translational processing of plants, with the rapid growth rate and potential for high-density culture of microorganisms (Walker et al., 2005a). Unicellular photosynthetic green algae are most commonly used for protein production as they only require inexpensive salt-based media, carbon dioxide and light for growth. Most green algae are also classified as generally regarded as safe (GRAS), making purification and processing of expressed products much less onerous for many targeted applications. Contrary to transgenic plants which must be strictly contained to avoid the transfer of transgenic material to surrounding wild-type flora by airborne vectors, microalgae can be cultivated in open facilities as no such transfer can occurs. On the economics side, based on recombinant antibody production studies, the cost of production per gram of functional antibody is $150, $0.05 and $0.002 (USD) in mammalian, plant and microalgal bioreactor systems respectively, making the latter system very economically attractive (Mayfield et al., 2003). A comparison of different recombinant protein expression systems is shown in Table 1.

Despite the recent surge of interest and successful transformation of a myriad of microalgal species, transgenic strains belonging to the *Chlamydomonas*, *Chlorella*, *Volvox*, *Haematococcus* and *Dunaliella* genera remain the most widely used and studied (Griesbeck et al., 2006; Raja et al., 2008; Rosenberg et al., 2008), and many obstacles remain to be overcome before microalgae can be considered standard expression systems. The large majority of current work is performed with *Chlamydomonas reinhardtii*, as it is the best characterized of the microalgal species. Considerable progress has been made in metabolic engineering towards increasing the expression of naturally produced compounds, with varying levels of success (Rosenberg et al., 2008). Although recombinant protein production is notably hindered by low expression levels, the continuing development of genetic engineering tools for microalgae has allowed the expression of fully functional antibodies (Franklin and Mayfield, 2005; Tran et al., 2009), therapeutics (Boehm, 2007; Weathers et al., 2010), and bactericides (Li and Tsai, 2009) at economically viable levels. Despite this progress, however, success essentially remains anecdotal and no wide-ranging system or protocol leading to high-level expression has been established.

Table 1
Comparison of different recombinant protein expression systems. Table modified and expanded from Walker et al. (2005a).

System	System characteristics							
	Molecular				Operational			
	Glycosylation	Gene size	Sensitivity to shear stress	Recombinant product yield	Production time	Cost of cultivation	Scale-up costs	Cost for storage
Bacteria	None	Unknown	Medium	Medium	Short	Medium	High	Low (−20 °C)
Yeast	Incorrect	Unknown	Medium	High	Medium	Medium	High	Low (−20 °C)
Insect[a]	Correct, but depends on strain and product	Limited	High	Medium to high	Long	High	High	High (liquid N_2)
Mammalian cells	Correct	Limited	High	Medium to high	Long	High	High	High (liquid N_2)
Plant cells	Correct[b]	Unlimited	N/A	High	Long	Low	Very low	Low (room temperature)
Unicellular microalgae	Correct[b]	Unlimited	Low	Generally low	Short	Very low	Low	Low (room temperature)

[a] (Ikonomou et al., 2003; Tomiya, 2009).
[b] Nuclear expressed proteins only, no glycosylation in chloroplast.

Excellent reviews on recombinant microalgae have already been published (León-Bañares et al., 2004; Walker et al., 2005a; Griesbeck et al., 2006; Mayfield et al., 2007). Given the rapid developments in transgenic microalgae technology, however, an update of recent progress in high-level expression of recombinant proteins is warranted. Transformation methods for both nuclear and chloroplast microalgal genomes, strategies to increase recombinant protein yields, and potential research directions of interest are here reviewed. A concise and up-to-date synthesis of these subjects should prove a useful reference to facilitate future work in the area of microalgal bioreactors.

3. Genetic transformation methods

The relatively recent study of transgenic microalgae was only made possible following the development of efficient techniques for the delivery of DNA to target microalgal genomes. These transformation methods have been briefly reviewed before (León-Bañares et al., 2004; Walker et al., 2005a; Griesbeck et al., 2006). Although some of these methods may not have significantly changed since their initial development, they are still being applied and studied in recent work. An updated list of available methodologies is compiled here.

3.1. Cell wall-deficient strains

Successful transformations were achieved in wild-type microalgae using all the methods described, although at very low efficiencies. The use of cell wall-deficient strains, or the removal of the cell walls from wild-type strains, greatly increases the number of transformants recovered following transformation.

Protocols for cell wall removal have been developed which facilitate the study of microalgae. These protocols involve the mating of mating type plus (mt+) and mating type minus (mt−) gametes of *C. reinhardtii*. The specific cell–cell recognition resulting from flagellar interaction leads to the release of enzymes, autolysin or lysin, that cause cell wall degradation. These enzymes can be purified and used as a pre-treatment to transformation. A detailed protocol for production and purification of these enzymes is given by Buchanan and Snell (1988) and a detailed study of the mating process was more recently reported by Hoffmann and Beck (2005).

3.2. Particle bombardment

Bombardment of target cells with DNA-coated metallic particles is a widespread, simple, effective and highly reproducible transformation method. This method has been successfully employed for the transformation of most standard cellular expression systems, and it is therefore not surprising that it is also useful for the study of microalgae. The main drawback of the particle bombardment method is the cost of the required specialized equipment. Although the number of transformants recovered following particle bombardment can be low, it remains the most effective method for the transformation of chloroplasts, as it allows for the delivery of multiple copies of recombinant DNA through both the cellular and chloroplast membranes, increasing the chance for a successful integration event to occur (Boynton and Gillham, 1993).

This method has been shown to be effective for the stable nuclear (Mayfield and Kindle, 1990) and chloroplast (Boynton et al., 1988; El Sheekh, 2000) transformation of *C. reinhardtii*, the transformation of *Volvox carteri* (Schiedlmeier et al., 1994), *Chlorella sorokiana* (Dawson et al., 1997), *Chlorella ellipsoidea* (Chen et al., 1998) and *Chlorella kessleri* (El-Sheekh, 1999) species, transient transformation of *H. pluvialis* (Teng et al., 2002) and the stable nuclear transformation of the diatom *Phaeodactylum tricornutum* (Apt et al., 1996). Recent work has shown that the particle bombardment method is also effective for the transformation of more complex algal species, such as the multicellular *Gonium pectorale* (Lerche and Hallmann, 2009).

3.3. Glass beads method

A simple and effective transformation method consists of agitating cell wall-deficient microalgal cells with recombinant DNA, polyethylene glycol (PEG), which greatly increases transformation efficiency, and glass beads. Despite the drop in cell viability to 25% following agitation with the beads, a nuclear transformation efficiency of 10^3 transformants/μg DNA was achieved using this method (Kindle, 1990) and an efficiency of 50 transformants/μg DNA was achieved for the transformation of *C. reinhardtii* chloroplasts (Kindle et al., 1990). Compared to the particle bombardment method, the glass beads method is simpler, more efficient for nuclear transformations, and much less expensive as it does not require specialized equipment. A recent study showed that the glass beads method is also more efficient than particle bombardment for the transformation of *Dunaliella salina* (Feng et al., 2009).

A similar protocol, using silicon carbon whiskers instead of glass beads to pierce cells, has also been used successfully (Dunahay, 1993; Wang et al., 1995). The cell viability following agitation is much improved, but due to low transformation efficiencies, high cost of materials, and health concerns associated with the handling of the whiskers, the glass beads are generally preferred.

3.4. Electroporation

The effectiveness of microalgal electroporation, or the induction of macromolecular uptake by exposing cell walls to high intensity

electrical field pulses, was first reported by Brown et al. (1991). Electroporation specifically disrupts lipid bilayers, leading to efficient molecular transport across the plasma membrane (Azencott et al., 2007). Efficient electroporation-mediated transformation was achieved in both wild-type and cell wall-deficient strains (Brown et al., 1991). The transformation efficiency of electroporation is two orders of magnitude higher than the glass beads method, and only requires relatively simple equipment (Shimogawara et al., 1998). Important parameters affecting the effectiveness of electroporation include field strength, pulse length, medium composition, temperature and membrane characteristics (Brown et al., 1991) as well as the concentration of DNA (Wang et al., 2007a).

Electroporation was successfully used for the transformation of *D. salina* (Geng et al., 2004; Sun et al., 2005; Wang et al., 2007b; Sun et al., 2008; Feng et al., 2009), *Dunaliella viridis* (Sun et al., 2006) and *Dunaliella tertiolecta* (Walker et al., 2005b) species, *C. reinhardtii* (Tang et al., 1995; Shimogawara et al., 1998; Kovar et al., 2002; Ladygin, 2003, 2004), *Chlorella* species (Chow and Tung, 1999; Wang et al., 2007a), and *Nannochloropsis oculata* (Chen et al., 2008; Li and Tsai, 2009).

3.5. Agrobacterium tumefaciens-mediated transformation

Transformation by the tumour-inducing *A. tumefaciens* is another efficient means of delivering genetic material, although this method has so far been mainly used to modify plant cells. Transformation results from the stimulation of cell division by products encoded by T-DNA transferred from *Agrobacterium* to the target cell. The T-DNA and virulence (*vir*) regions are located on the tumour inducing plasmid (pTi). The *vir* system processes and transfers any DNA between the short flanking repeats that delimit the T-DNA, making *Agrobacterium* an efficient DNA delivery system (Akhond and Machray, 2009).

Using the *Agrobacterium*-mediated transformation method, *C. reinhardtii* was successfully transformed with *uidA* (β-glucuronidase), *gfp* (Green Fluorescent Protein) and *hpt* (hygromycin phosphotransferase) reporter genes, with a fifty-fold increase in resulting transformants compared to the glass beads method (Kumar et al., 2004). GUS (β-glucuronidase), *gfp* and *hpt* genes were also successfully integrated in *H. pluvialis* using this method (Kathiresan et al., 2009). Although results are currently sparse regarding transformation of microalgae with *Agrobacterium*, the method holds considerable promise, and given its success in plant transformations warrants further study. A detailed protocol for the transformation of *H. pluvialis* was devised and studied by Kathiresan and Sarada (2009).

3.6. Gene copy number

As further discussed in subsequent sections, expression levels of foreign genes in microalgae, in addition to generally being low, are inconsistent and difficult to predict. A significant part of this variation in expression levels arises from inconsistencies in the number of transgene copies integrated within a particular genome. Multiple gene integration patterns, notably complex ones such as inverted or tandem repeats, are associated with low-level transgene expression. The reduction in expression levels is due to homology-dependent gene silencing (HDGS), which affects expression when multiple copies are present, whether at a single locus or at unlinked sites. Single-copy transformants, which generally have higher and more predictable expression levels, are therefore desirable. Silencing occurs at the transcriptional or post-transcriptional level, and is believed to have arisen as a defense mechanism of plants against viruses and as a means of regulating gene expression. Several reviews on transgene silencing and related expression variation in plants have previously been published (Muskens et al., 2000; Baulcombe, 2004; Butaye et al., 2005; Angaji et al., 2010; Teixeira and Colot, 2010; Marenkova and Deineko, 2010).

The transformation method used may impact the number of integrated transgene copies in target cells, although given the variability of reported results, the number of transgene integrations may depend on numerous factors. Although direct DNA-transfer methods such as particle bombardment or glass bead shearing generally lead to a large number of integrated gene copies which may increase silencing effects, by varying the amount of delivered DNA, single or low number copy integration can be achieved, making these methods fairly flexible (Yao et al., 2006; Jayaraj et al., 2008; Lowe et al., 2009). The number of transgene copies integrated following electroporation-mediated transformation is highly variable, but this transformation method has been shown to lead to low-copy transformants. In addition to the electroporation conditions discussed previously, copy integration also depends on the quality of the target tissue and pretreatments it may undergo prior to transformation (Sorokin et al., 2000). *Agrobacterium*-mediated transformation generally leads to low copy number integration, and leads to a higher proportion of single-copy transformants (Butaye et al., 2005; Wu et al., 2008; Zale et al., 2009; Oltmanns et al., 2010).

4. Selection markers

Transformation protocols are useful only if effective markers are available to select successful transformants. The majority of selectable markers confer a resistance to antibiotics or operate by complementation of metabolic mutants. In the former method, genes conferring antibiotic resistance, whether hybridized to the foreign DNA of interest or co-transformed on a distinct vector, are most commonly used for selection of microalgal transformants. The other strategy relies on metabolic or photosynthetic rescue of microalgal mutants with wild-type gene constructs, with transformant selection protocols based on cultivation conditions. The latter method may be particularly useful for chloroplast transformations, where integration of genetic material occurs by homologous recombination. In such cases, hybrid foreign DNA constructs containing wild-type genes can not only rescue microalgal mutants in which that gene is knocked-out, thus allowing for selection, but specifically targets adjacent regions for foreign DNA integration.

Although lists of selectable markers in microalgae have been compiled in past reviews (León-Bañares et al., 2004; Walker et al., 2005a; Griesbeck et al., 2006), novel markers have since been developed.

Phytoene desaturase (PDS), a rate-limiting enzyme involved in the production of carotenoids by green algae, is inhibited by certain herbicides. The induction of point-mutation in PDS makes the transgenic strain resistant to the herbicide norflurazon, and this has been shown to be an effective selectable marker in *H. pluvialis* (Steinbrenner and Sandmann, 2006) and *Chlorella zofingiensis* (Huang et al., 2008).

The *ARG9* gene from *C. reinhardtii* encodes a plastid *N*-acetyl ornithine aminotransferase, an enzyme involved in arginine synthesis. The integration of an *ARG9* cassette in the plastid chromosome of the nuclear *arg9* mutant restores arginine prototrophy, making ARG9 a novel selectable marker for plastid transformations (Remacle et al., 2009).

Microalgal chloroplasts present an attractive platform for the expression of recombinant therapeutic or nutritional products at high levels, making marker-free systems highly desirable in such applications. Marker genes are engineered for high level expression for effective protection of cells for selection. Once homoplastomic transformation is achieved, 5–18% of the total soluble protein can consist of marker gene products, which lowers the maximum yield of the target protein. If the recombinant algae are destined for human or animal consumption, unnecessary DNA, including genes conferring resistance to antibiotics, is undesirable. Marker removal can be achieved by homology-based excision, excision by phage site-specific

recombinases, transient cointegration of the marker gene or the cotransformation–segregation approach. An excellent review of these processes is provided by Lutz and Maliga (2007).

5. Nuclear versus chloroplast genomes

Although recombinant protein expression in the microalgal nuclear, mitochondrial and chloroplast genomes has been achieved, commercially viable expression levels have only been reported in the latter, and for relatively simple proteins.

Nuclear expression of foreign proteins remains very low, for reasons that are as yet not well understood. Positional effects, RNA silencing, a prohibitively compact chromatin structure and non-conventional epigenetic effects have been proposed as possible causes. The latter is supported by a recent study describing a protocol for the selection of highly expressed nuclear transgenes following UV-induced mutations of transformed strains. Using this protocol, yields of foreign proteins accounting for 0.2% total soluble protein (TSP) were achieved, which is relatively high for nuclear expression (Neupert et al., 2009). Like all eukaryotic genomes, post-transcriptional and -translational processing is performed, and post-translational targeting to specified downstream organelles is possible, making nuclear expression necessary for complex protein expression despite low yields.

Chloroplasts are generally preferred for foreign protein expression in microalgae due to high-expression levels and, contrary to nuclear transformation, the possibility of targeted insertion of sequences by homologous recombination. Based on plant studies, chloroplast proteolytic pathways are limited, and the chloroplast envelope may protect foreign protein from degradation, thus increasing their overall yield (Faye and Daniell, 2006). The highest reported protein expression level in chloroplasts is slightly over 10% TSP (Surzycki et al., 2009) although the large majority of yields are around 5% TSP and lower (Manuell et al., 2007).

6. Factors affecting protein expression and strategies for its increase

The development of economically viable microalgal expression systems is currently hindered by low and inconsistent recombinant protein yields. Recent efforts towards yield improvement have concentrated, usually as independent parameters, on the study of promoters, UTR sequences and fusion between native and recombinant peptides in microalgal chloroplasts. The regulation of recombinant protein expression is a complex system consisting of interacting elements. Although the extent of interdependence between different factors is not completely understood, several strategies and mechanisms of particular interest have proven to increase recombinant protein yields in microalgae. Although the systematic study of some of these factors has historically been prohibitively time consuming, the ongoing shift from traditional gene isolation from known organisms and subsequent modifications to the *de novo* design and synthesis of genes provides us with new strategic avenues for the design of time-efficient and comprehensive studies (Wu et al., 2007). Recent progress and development on key factors affecting recombinant protein yields are reviewed here.

6.1. Codon optimization

It is well established that the genomes of different organisms, and the different genomes of single organisms, employ codon biases as mechanisms for optimizing and regulating protein expression (Gustafsson et al., 2004). As is the case for most heterologous genes, optimizing the codon usage of microalgae-destined transgenes to reflect this bias increases their expression efficiency by increasing their translation rates, and may decrease their susceptibility to silencing (Heitzer et al., 2007). In prokaryotic genomes, such as those from microalgal chloroplasts, codon bias is the single most important determinant of protein expression (Lithwick and Margalit, 2003; Surzycki et al., 2009), and adjustment of codons in transgenes is necessary for high level (i.e. commercially viable) expression (Franklin et al., 2002; Mayfield et al., 2003; Mayfield and Schultz, 2004).

The Codon Adaptation Index (CAI) is used as a quantitative tool to predict heterologous gene expression levels based on their codon usage. As the chloroplast, mitochondrial and nuclear genomes of microalgae may exhibit different codon biases, as is the case of *C. reinhardtii*, genome-specific CAI values should be used for optimal translational.

Underscoring the importance of codon optimization in biotechnological applications, several free software and web applications have recently been developed to estimate CAI values and optimize the codon usage of sequences. E-CAI (http://genomes.urv.es/CAIcal/E-CAI) determines whether differences in CAI between sequences are significant or arise from biases in G + C or amino-acid composition (Puigbò et al., 2008a). The online OPTIMIZER application (http://genomes.urv.es/OPTIMIZER) optimizes the codon usage of provided sequences using pre-constructed usage tables based on either a 'one amino acid-one-codon' basis, Monte-Carlo algorithms or a novel algorithm for optimization with minimal changes (Puigbò et al., 2007). CAI-cal (http://genomes.urv.es/CAIcal) provides an integrated set of tools for the optimization of codon usage (Puigbò et al., 2008b). Some gene design software packages such as *Gene Composer* include gene optimization functionalities (Lorimer et al., 2009). A list of older codon optimization software has been compiled by Villalobos et al. (2006). A database of CAI and codon usage indices for most sequenced species is available online at http://www.kazusa.or.jp/codon/ (Nakamura et al., 2000).

Codon optimization is an effective and necessary step in gene sequence optimization, and one relatively simple to address with recent advances in DNA synthesis technology, but it is not the only factor to be considered. An excellent review by Welch et al. (2009) describes important factors and useful strategies for the *de novo* design of genes optimized for recombinant protein production.

6.2. Transformation-associated genotypic modifications

Transgenes are inserted in the chloroplast genome by homologous recombination, which implies that each transformant obtained should be identical if using a single integration vector. Identical recombinant protein expression profiles for each transformant are therefore expected.

Surzycki et al. (2009) however, have observed protein yields varying from 0.88% to 20.9% total cell protein (TCP), the latter being the highest yield reported to date, in transgenic lines obtained from a single biolistic transformation. They associated this variation to genotypic modifications resulting from the transformation process, dubbed *transformosomes*. The observed expression levels of transgenic proteins, which are to date low, may therefore depend more on these modifications than on the selection of promoters, UTRs or insertion sites. Although the mechanisms behind transformosomes are as yet unconfirmed, they may be due to additional insertions of the transgenic gene in the nuclear genome, which would interfere with proteins regulating recombinant yields (Surzycki et al., 2009). Nuclear insertion may also interfere with the expression of chloroplast-bound genes necessary for photosynthesis or with proteins involved in their targeting and transfer. Extensive screening of transformants is thus recommended to isolate the most productive ones for further process development.

6.3. Endogenous enhancer and regulatory elements

Inserting introns from native genes in heterologous sequences under the control of that gene's promoter has been shown to increase protein

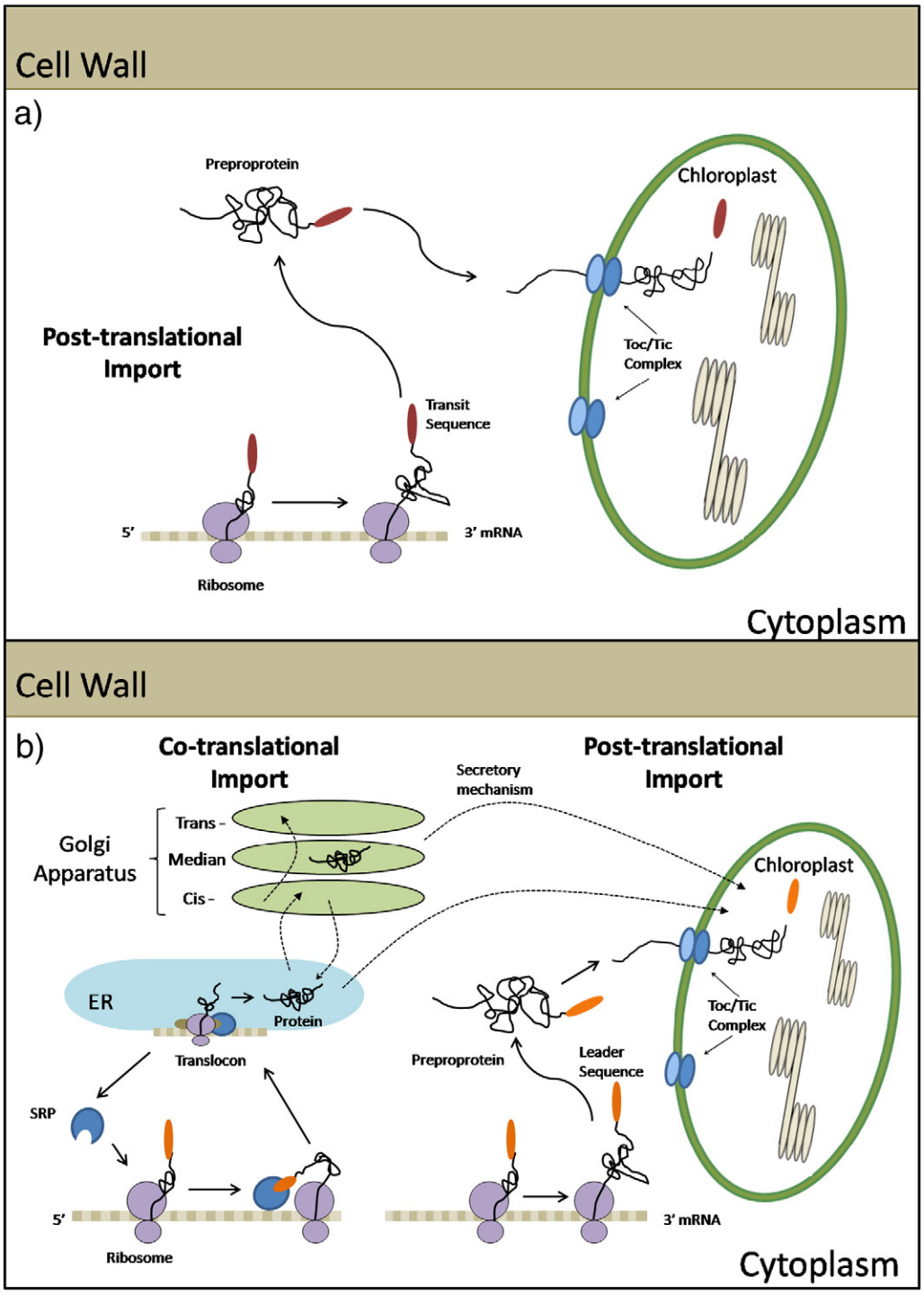

Fig. 1. Main chloroplast protein import mechanisms in plants. (a) Classic post-translational import mechanism. (b) Simultaneous co-translational and post-translational mechanisms. Additional work is required to determine if these pathways are reproduced in microalgae and if they can be utilized for protective storage of recombinant proteins.

yields. A recent study by Eichler-Stahlberg et al. (2009) showed that inserting three introns from native *C. reinhardtii* RBCS2 chloroplast gene in recombinant codon-optimized luciferase and erythropoietin increased expression upwards of 400% compared to base levels. Although each individual RBCS intron had a positive effect on expression, their integration in their physiological order and number produced a synergetic effect. Expression of recombinant genes in the nuclear genome of *C. reinhardtii* also improved following the insertion of the first RBCS2 intron, which has been shown to contain an enhancer element (Lumbreras et al., 1998; Berthold et al., 2002).

A number of *C. reinhardtii* chloroplast gene products regulate the translation of their own mRNA through feedback inhibition (Wostrikoff et al., 2004; Minai et al., 2006). This may partially explain the low expression levels of heterologous genes in microalgal chloroplasts compared to tobacco chloroplast expression systems, in which this inhibition is not observed. Manuell et al. (2007) showed that the product of the endogenous *psbA* chloroplast gene, D1 protein, does inhibit the expression of recombinant M-SAA under the control of the psbA 5′UTR. In this case, however, competition between endogenous and recombinant psbA-mediated constructs for limited transcription

and translation factors reduced protein accumulation. They report a maximum yield of just over 5% TSP.

The fusion of recombinant products to native proteins has also resulted in an increase of protein yield. Muto et al. (2009) fused the endogenous Rubisco LSU protein to a recombinant luciferase through a cleavable domain. This resulted in a 33-fold increase in luciferase expression compared to luciferase expressed alone, and near-wild-type Rubisco expression levels. These results indicate that recombinant protein accumulation in algal chloroplasts can be enhanced by fusion with a native protein. The usefulness of engineering proteolytic processing sites to liberate recombinant proteins from the native ones has also been demonstrated, which would simplify product purification in such applications.

6.4. Sensitivity to proteases

The level of foreign protein accumulation results from a balance between rates of protein synthesis and degradation, the latter of which is increasingly found to impact recombinant product yields. Proteolytic enzymes, which are essential for endogenous protein processing, may lead to the degradation of foreign proteins after synthesis, or interfere with their correct assembly and post-translational modification. Proteolysis may also lead to inconsistent results and to difficulties in downstream processing or purification due to degraded or non-functional protein fragments. Only limited information on the impact of degradation on yields in microalgae is available, but many studies on proteolysis in plants have been reported. In *C. reinhardtii*, proteolytic degradation is one of the principal factors affecting recombinant protein yield (Surzycki et al., 2009).

Several strategies are available to minimize proteolytic degradation of foreign proteins in plants, which may also be applicable in microalgal systems. The more interesting of these strategies are briefly reviewed here, although additional work is required to evaluate their effectiveness in microalgal systems specifically. An excellent review of proteolytic degradation in plants has been published by Doran (2006).

For nuclear-expressed proteins, degradation can be minimized by targeting protein synthesis to the ER rather than to the cytosol (Conrad and Fiedler, 1998), a strategy that lead to a 10^4-fold increase in recombinant growth factor expression in tobacco (Wirth et al., 2004). As the plant cell ER contains very few proteases, in applications where protein secretion or modification in the Golgi are not required, proteins can be retained in the ER using KDEL or HDEL sequences, which may have the added effect of enhancing proper folding and stability of certain proteins (Nuttall et al., 2002). Alternatively, the co-expression of protease inhibitors has proven useful in increasing recombinant protein yields in plants, without affecting normal growth and development (Van der Vyver et al., 2003).

Proteins not requiring post-translational modifications can be expressed in the chloroplasts of algae. In plant chloroplasts, proteolytic pathways, although present and necessary for processing, are limited, and may not interfere with recombinant protein accumulation (Bock, 2001). Chloroplasts may therefore serve as a protective envelope for expressed foreign proteins during long-term storage.

Many proteins do require post-translational modifications not performed in the prokaryotic plastids. The large majority of plant chloroplast proteins are encoded in the nuclear genome and imported from the cytoplasm (Faye and Daniell, 2006; Jarvis, 2008). A possible strategy to minimize proteolytic degradation of proteins requiring modification would therefore be to target the nuclear-expressed proteins to the chloroplast for storage. The simplified chloroplast protein import mechanisms are illustrated in Fig. 1. According to the classic, and dominant, mechanism, chloroplast proteins synthesized by cytosolic ribosomes are targeted to the chloroplast through interactions between an N-terminal transit sequence and the *Toc* and *Tic* complexes in the chloroplast membranes (Fig. 1a). In addition to the post-translational targeting of proteins to chloroplasts by this mechanism, certain proteins (e.g. CAH1 and NPP1) are also co-translationally synthesized on membrane-bound ribosomes and inserted in the ER through N-terminal signal peptides (Levitan et al., 2005). These proteins can then be targeted to the chloroplast directly or undergo further processing in the Golgi apparatus prior to delivery to the chloroplast via the secretory pathway (Fig. 1b) (Villarejo et al., 2005; Radhamony and Theg, 2006; Nanjo et al., 2006; Kitajima et al., 2009; Hummel et al., 2010). Although the existence of this mechanism is well supported by experimental data, specific details on endomembrane-mediated chloroplast targeting and import mechanisms remain to be fully elucidated. A more detailed account on this subject is outside the scope of this paper, but additional details are provided in excellent reviews by Faye and Daniell (2006); Inaba and Schnell (2008); Jarvis (2008), and Faso et al. (2009).

7. Conclusions and future prospects

The complex interplay of many factors, including enhancer elements, regulatory mechanisms, competition for available transcription and translation factors, codon dependency, transformation-associated events, sensitivity to proteases, protein localization and gene silencing, underscores the difficulties in establishing a standard system for recombinant protein production in microalgae. Although the systematic study and optimization of some of these parameters is, sometimes prohibitively, difficult and time consuming using traditional methods, as the *de novo* gene design and synthesis become less expensive and more readily available, the design of more efficient experiments will become possible.

The factors discussed in the present paper deal with the 'upstream' side of process engineering and design. Once transgenic strains are developed, the cultivation operating parameters, including growth media composition, temperature, pH, CO_2 concentration, agitation and illumination, can significantly affect protein yields, whether directly through molecular effects on cellular mechanisms, or indirectly through their effect on microalgal growth and maximum cell density. Cooperation among researchers on both upstream and downstream bioprocess design will further facilitate the development of economically viable transgenic microalgae-based bioreactors.

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