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Chlamydomonas reinhardtii chloroplasts as protein factories

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Protein-based therapeutics are the fastest growing sector of drug development, mainly because of the high sensitivity and specificity of these molecules. Their high specificity leads to few side effects and excellent success rates in drug development. However, the inherent complexity of these molecules restricts their synthesis to living cells, making recombinant proteins expensive to produce. In addition to therapeutic uses, recombinant proteins also have a variety of industrial applications and are important research reagents. Eukaryotic algae offer the potential to produce high yields of recombinant proteins more rapidly and at much lower cost than traditional cell culture. Additionally, transgenic algae can be grown in complete containment, reducing any risk of environmental contamination. This system might also be used for the oral delivery of therapeutic proteins, as green algae are edible and do not contain endotoxins or human viral or prion contaminants.

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Introduction

Recombinant proteins are used in a variety of healthcare, scientific and industrial applications, and the use of these molecules will continue to grow as new proteins are discovered and engineered. Today, recombinant proteins are expressed in several production systems including bacteria, yeast, insect and mammalian cell culture, and many plant species. Each system has limitations as well as distinct advantages in terms of the type of protein that can be made, the quantity that can be produced, ease of manipulation, and cost of operation [1].

The aim of this review is to examine progress on the expression of recombinant proteins in the chloroplast of the single-celled eukaryotic alga, *Chlamydomonas*

reinhardtii. Even though this green alga has served as a model organism for much of our current understanding of chloroplast function, from the mechanisms of gene regulation to assembly and function of the photosynthetic apparatus, only recently has work begun on the generation of transgenic algae for the expression of recombinant proteins. Expressing therapeutic proteins in micro-algae has several advantages over other systems employed today. First, chloroplasts have been shown to correctly fold and assemble complex mammalian proteins [2**], and the length of time required from the initial transformation event to having usable (milligram to gram) quantities of a protein can be relatively quick in algae. Stable transgenic lines can be generated in as little as 10 days, and scale-up to production volumes can potentially be achieved a few weeks after this (Figure 1). In addition, algae can easily be grown in full containment reducing any concern about environmental contamination of the therapeutic protein. Moreover, because algae do not have pollen there is no potential for the introduction of transgenes into food crops, as potentially could occur in higher plants by gene flow (via pollen) to surrounding plants [3]. Finally, many green algae fall into the category of Generally Recognized as Safe (GRAS), meaning they are safe to eat, and are therefore potentially a source for the oral delivery of therapeutic proteins, perhaps with little or no purification. The potential of eukaryotic algae as a production platform for recombinant proteins with therapeutic and other uses will be discussed.

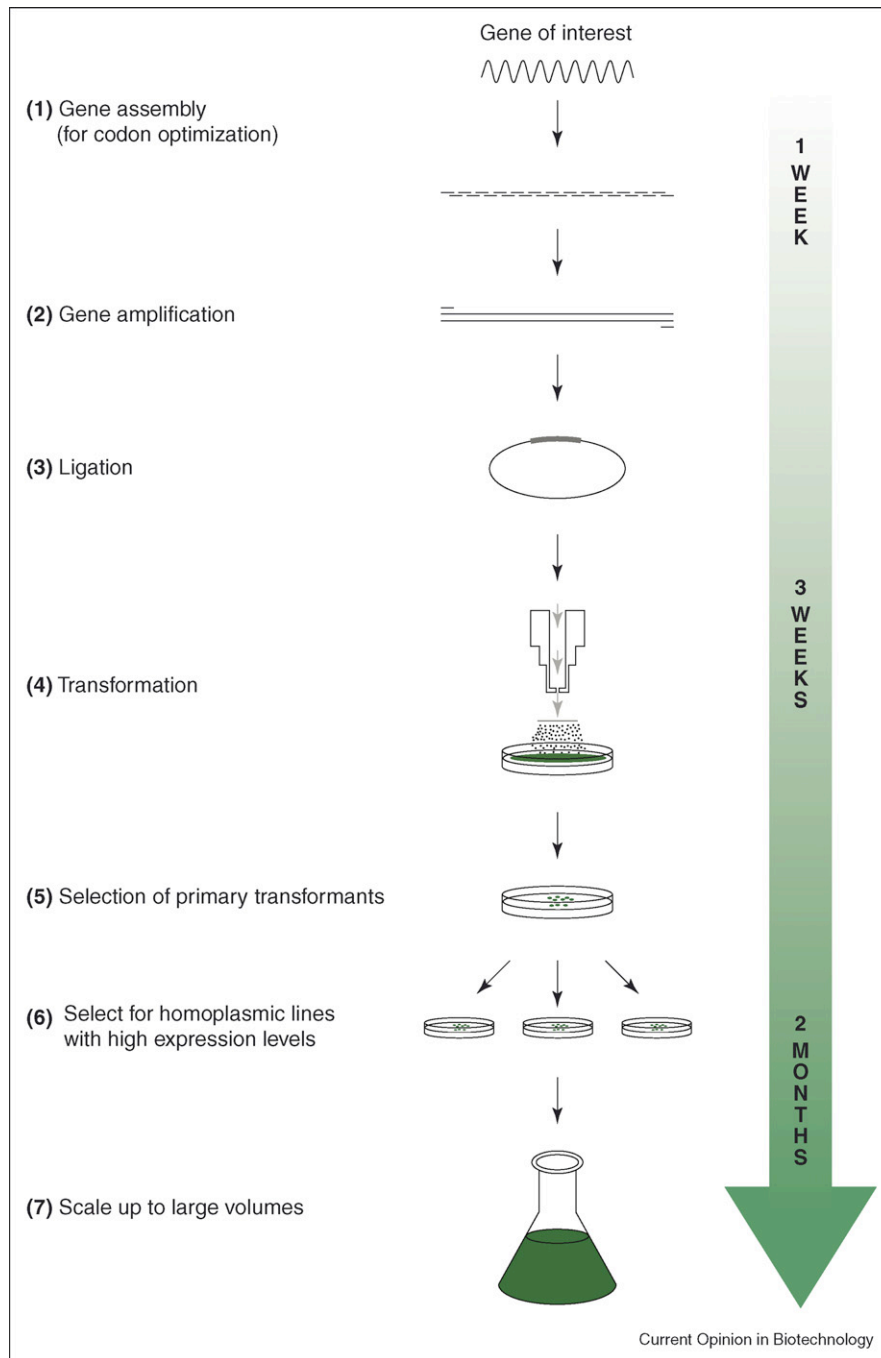
First attempts to express recombinant proteins in *C. reinhardtii*

Stable chloroplast transformation was first accomplished in 1989 when Boynton and co-workers restored the photosynthetic capacity of a *C. reinhardtii* mutant by cell bombardment with high-velocity microprojectiles coated with the wild-type gene [4]. A similar achievement in higher plants was made two years later by introducing a mutated version of the 16S rRNA conferring spectinomycin and streptomycin resistance into *Nicotiana tabacum* chloroplasts [5]. Initial goals with this transformation technology were mainly focused on basic studies to determine the role of chloroplast genes and their regulatory sequences, and involved the use of reporter genes and antibiotic resistance genes for transformant selection.

The first attempts to express foreign proteins in the chloroplast of *C. reinhardtii* involved the bacterial neomycin phosphotransferase [6] and β -glucuronidase genes [7], both driven by *C. reinhardtii* chloroplast promoters.

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Figure 1



Stages for the production of recombinant proteins in *C. reinhardtii*. (1) *De novo* synthesis of the gene coding region with overlapping oligonucleotides to optimize the gene coding region to match the codon usage of *C. reinhardtii* chloroplast protein-coding sequences. (2) Amplification of the complete codon-optimized gene. (3) Ligation of the optimized gene into a vector containing DNA sequences homologous to regions of the *C. reinhardtii* chloroplast genome to target recombination. (4) Co-transformation of the vector with a selectivity vector into *C. reinhardtii* spread on a plate of selective medium. (5) Selection of primary transformations that appear on the antibiotic-containing medium. (6) Isolation of homoplasmic high recombinant protein expressing lines through continued selection on antibiotic media. (7) Growth of the high recombinant protein expressing line in large quantities of liquid media. The arrow on the right depicts the approximate time required for each step of the process.

These studies showed stable accumulation of recombinant mRNAs, but no protein accumulation could be detected. Using chloroplast promoters and 5' untranslated regions (UTRs) to drive the expression of the bacterial *aadA* gene, encoding aminoglycoside adenine transferase (AAD), stable accumulation of a foreign protein in transgenic chloroplasts was first reported in 1991: the transformed cells were able to resist spectinomycin treatment and an enzymatic assay detected AAD activity [8]. The first identification of foreign protein expression using western blot analysis was shown in 1999 for the bacterial β -glucuronidase reporter protein driven by either the *rbcL*, *psbA* or *atpA* promoters and 5' UTRs [9]. Similarly, expression of *Renilla* luciferase protein was also shown by western blot analysis in 1999 [10]. Although protein accumulation in each of these initial studies appeared to be very low, probably less than 0.01% of total soluble protein (TSP), the recovery of enzymatic activity of the recombinant proteins in all cases indicated that properly folded foreign proteins could be expressed in chloroplasts of *C. reinhardtii*.

Increased expression through codon optimization

As described above, early attempts to express foreign genes in *C. reinhardtii* chloroplasts generally resulted in no, or very low, accumulation of the desired recombinant protein [6,7,9]. All of these recombinant genes were directly taken from other systems and none was optimized for expression in *C. reinhardtii* chloroplasts. The genetic code is made up of many redundant triplets that encode for the same amino acid; however, it has been shown in a variety of organisms that certain codons are more frequently used than others [11]. The chloroplast of *C. reinhardtii* displays such codon bias, with codons containing adenine or uracil nucleotides in the third position favored over those with guanine or cytosine [12].

To directly test whether codon optimization could impact upon recombinant protein accumulation in *C. reinhardtii* chloroplasts, a green fluorescent protein (*gfp*) reporter gene was synthesized *de novo* in accordance with such codon bias. Codon-optimized *gfp* and non-optimized native *gfp* genes, both driven by the same promoter and UTR, were transformed into chloroplasts and recombinant protein expression was assayed [13]. Quantitation of GFP accumulation in these transgenic lines showed that the codon-optimized *gfp* gene resulted in an 80-fold increase in GFP accumulation compared with the non-optimized version. Subsequent studies using a codon-optimized human antibody gene [2**] and codon-optimized luciferase genes [14*] confirmed that codon bias plays a significant role in protein accumulation in *C. reinhardtii* chloroplasts. Increased protein production in these strains highlights the necessity for codon optimization of any gene for which high levels of protein production are desired.

Promoters and UTRs for increased protein expression

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. Transcriptional efficiency, transcript stability, and translational efficiency have all been shown to impact the expression of endogenous proteins in *C. reinhardtii* chloroplasts, and the 5' UTR of chloroplast mRNAs has key roles in each of these events. Transcriptional efficiency was shown to be regulated by both chloroplast gene promoters as well as sequences contained within the 5' UTR [15]. The most common promoter found in *C. reinhardtii* chloroplast genes is similar to bacterial σ^{70} -type promoters, containing bacterial-like -10 (TATAA-TAT) and -35 elements [16]. Additionally, sequences found within the 5' UTR are also important for optimal levels of transcription, although the nature of these internal enhancer sequences has yet to be defined [15]. Elements within both the 5' and 3' UTRs have also been shown to effect transcript stability [17*,18*], probably through interaction with *trans*-acting protein factors [19–21]. A variety of studies have revealed that translational efficiency is a rate-limiting step for chloroplast gene expression [22], and have shown that the 5' UTRs of plastid mRNAs contain key elements for translational regulation [23].

A combinatorial approach was used to directly assess the effect of various endogenous UTRs on recombinant protein expression in *C. reinhardtii* chloroplasts [24**]. Using a *gfp* reporter gene and the 5' and 3' UTRs from five different chloroplast genes a series of chimeric genes was created and integrated in the chloroplast genome. The highest levels of recombinant protein expression were obtained using either the *atpA* or *psbD* 5' UTRs, while no expression was observed using the 16S rRNA 5' UTR. The nature of the 3' UTR invariably had little effect on reporter protein accumulation [24**]. The GFP protein never accumulated to levels approaching those of endogenous chloroplast proteins, suggesting that sequences within the coding region, in addition to codon bias, might be important for translation efficiency. These results demonstrated that different chloroplast 5' UTRs support different levels of protein synthesis, and show that recombinant chimeric mRNAs are not translated as efficiently as endogenous native mRNAs in *C. reinhardtii* chloroplasts. The reasons why chimeric mRNAs are translated less efficiently than endogenous mRNAs are not clear at present.

Regulated expression of recombinant proteins in chloroplasts

Chloroplast gene expression is primarily post-transcriptionally regulated, during mRNA processing, stabilization, and predominantly, translation [22,25]. The translation of

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many chloroplast mRNAs is stimulated by light, with the highest level of light induction being for the *psbA* mRNA (which encodes D1, a core protein of photosystem II) [26]. In *C. reinhardtii*, light-activated translation of D1 is correlated with the association of a nuclear-encoded protein complex (consisting of RB47, RB38, RB60 and RB55) with the 5' UTR of the *psbA* mRNA [27]. Previous *in vitro* studies have shown that binding of the RB47/RB38/RB60/RB55 complex to the *psbA* 5' UTR is inactivated by ADP-dependent phosphorylation and oxidative disulfide-bond formation and activated by dephosphorylation and disulfide-bond reduction [28,29]. Light is proposed to regulate the phosphorylation and redox state of the protein complex via the photosynthetic electron transport chain [30].

Our laboratory has shown that the *psbA* 5' UTR is capable of conferring light-regulated translation to recombinant mRNAs [14*,24**]. This offers the potential to regulate recombinant protein expression, which might be necessary to express proteins that are not well tolerated by the chloroplast. We have also shown that *psbA*-driven heterologous protein expression is increased when the endogenous *psbA* gene is deleted [14*] (see also Update). Increased expression in the absence of the *psbA* gene product could be due to either the removal of auto-attenuation from D1 protein feedback [31**] or to reduced competition with endogenous *psbA* for limited transcription or translation factors. Further studies identifying the specific *cis*-elements in the *psbA* mRNA responsible for light-activation and auto-attenuation could allow for the generation of light-activated transgenes that are no longer inhibited by the presence of the native *psbA* mRNA or protein.

Other strategies for inducible expression of recombinant proteins in higher plant chloroplasts have been reported. These approaches place the chloroplast transgene under

the transcriptional regulation of an inducible factor that is co-introduced to the nuclear genome (e.g. T7 polymerase [32–34]) or to the chloroplast genome (e.g. the *lac* repressor [35]). Although neither of these strategies has been implemented in *C. reinhardtii* chloroplasts, there are no biochemical or molecular reasons why they would not work as well in algae as they do in higher plants. The ability to regulate recombinant protein expression in the chloroplast could be essential for the production of certain types of recombinant proteins, including those that would normally be lethal to the host cell [34].

Expression of therapeutic proteins in *Chlamydomonas* chloroplasts

To date, only a handful of therapeutic proteins have been expressed in algal chloroplasts (Table 1). The first of these was a human antiherpes antibody that contained an entire heavy chain coding region linked to a light chain variable region via a flexible peptide linker [2**]. The gene encoding this large single-chain antibody (HSV8-lsc) was synthesized in chloroplast codon bias and expressed using either the *atpA* or *rbcL* promoters and 5' UTRs. HSV8-lsc protein accumulated to approximately 0.5% TSP, and was found completely in the soluble phase with no apparent accumulation as insoluble aggregates. Moreover, the HSV8-lsc antibody correctly assembled as a dimer and was capable of binding its target antigen, herpes simplex viral coat protein. These data established that algal chloroplasts have the capacity to synthesize fairly complex molecules in a soluble and active form. A simplified version of this antibody, HSV8-scFv, has also been expressed in the *C. reinhardtii* chloroplast. This scFv contained only the variable regions of the HSV8 light and heavy chains joined by a peptide linker. The HSV8-scFv accumulated to slightly higher levels than HSV8-lsc, and again was completely soluble and able to bind herpes viral coat protein in enzyme-linked immunosorbent assays (ELISA).

Table 1

Recombinant proteins produced in *C. reinhardtii* chloroplasts.

Protein product	Application/comments	Reference
Aminoglycoside adenine transferase	Reporter, confers spectinomycin and streptomycin resistance	[8]
β -Glucuronidase	Reporter, catalyzes the conversion of substrates to colored products	[9]
Renilla luciferase	Reporter protein, luminescent	[10]
Aminoglycoside phosphotransferase	Reporter, confers kanamycin and amikacin resistance	[49]
Green fluorescent protein	Reporter protein, fluorescent	[13]
HSV8-lsc	Pharmaceutical, first mammalian protein expressed	[2**]
Cholera toxin B subunit fused to foot and mouth disease VP1	Pharmaceutical, vaccine	[36**]
Bacterial luciferase	Real-time reporter protein, can be visualized in living cells	[14*]
HSV8-scFv	Pharmaceutical, classic single-chain antibody	[50]
Allophycocyanin	Fluorescent protein	[48**]
Human metallothionein-2	Pharmaceutical, UV protection	[38]
Firefly luciferase	Real-time reporter protein, can be visualized in living cells	[51**]
Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Pharmaceutical	[37]

The potential use of *C. reinhardtii* chloroplasts as a platform for vaccine production has also been tested. With this purpose in mind, a gene containing the foot and mouth disease virus (FMDV) VP1 coding region fused to the cholera toxin B (CTB) chain coding sequence was expressed in the *C. reinhardtii* chloroplast [36^{••}]. Although none of the coding region was codon-optimized, the chimeric protein was detected by western blot analysis and ELISA with yields reported at up to 3% TSP. The fusion protein was also reported to have both GM1-ganglioside binding affinity and antigenicity for both the VP1 and CTB proteins [36^{••}].

Another potential therapeutic protein, the human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has also been expressed in *C. reinhardtii* chloroplasts [37[•]]. The C-terminal soluble portion of TRAIL is sufficient to induce selective apoptosis in cancerous and virus-infected cells. Western blot analysis showed that soluble TRAIL was expressed in chloroplasts, and the levels of protein expression were estimated to be about 0.5% TSP. No bioactivity data were reported for this protein. Finally, a human metallothionein-2 protein has been reported to be expressed in *C. reinhardtii* chloroplasts [38]. This protein has the potential for reducing DNA damage caused by UV irradiation. Expression of the human cDNA was driven by the *chlL* promoter and UTR and expression of the protein in plastids was found to confer UV resistance to the transformed cells. The expression of this set of recombinant proteins, although small, shows the potential of algal chloroplasts for the production of human therapeutic proteins.

Post-translational modification and proteolysis in the chloroplast

Similar to bacteria, chloroplasts lack the machinery to perform complex post-translational modifications, like glycosylation, on proteins. Many mammalian proteins, including antibodies, are normally glycosylated during their synthesis and export from the mammalian cell, and thus it is important to understand the activity of non-glycosylated proteins produced in chloroplasts. Carbohydrate moieties added to proteins during glycosylation can effect assembly, serum half-life, and the function of antibodies [39]. Non-glycosylated antibodies show differential effects on various antibody activities, and have greatly reduced activation of complement and somewhat reduced Fc-mediated binding in activation of antibody-dependent cell-mediated cytotoxicity (ADCC) [39]. Complement fixation and ADCC activation are not required for antigen binding, and therapeutic antibodies that function to sequester molecules or block binding sites do not require the activation of ADCC and strive to avoid activation of complement. Thus, chloroplast-expressed non-glycosylated antibodies might actually be superior to glycosylated antibodies for some therapeutic uses.

Another post-translational modification required for the proper function of antibodies, as well as many other eukaryotic proteins, is disulfide-bond formation. *Escherichia coli* do not form structural disulfide bonds in proteins that accumulate in their cytoplasm [40], and hence bacteria are not an ideal system for the expression of proteins that require disulfide bonds. Expression of human somatotropin in tobacco chloroplasts [41] provided the first evidence of disulfide-bond formation in a plastid-expressed recombinant protein. Since then, many antibodies and other proteins have been expressed in chloroplasts of both higher plants and *C. reinhardtii* and have been shown to contain the expected disulfide bonds (see [42] for a review from higher plants). Thus, chloroplasts could be an excellent system for the expression of proteins that require structural disulfide bonds. The chloroplast proteins responsible for disulfide-bond formation could be the same proteins used to transduce the light activation signals used to regulate chloroplast translation, as one of these redox-dependent proteins is a protein disulfide isomerase [43].

It has also been recognized, particularly for antibodies, that proteolysis of recombinant proteins can be a major factor in protein accumulation [44[•]]. Proteolysis not only reduces yield but also creates heterogeneity in proteins that can complicate downstream purification. Chloroplasts of both higher plants and algae contain proteases commonly found in bacteria; Clp, Deg and FtsH proteases are all found in the nuclear genome of *C. reinhardtii* with at least one ortholog of each targeted to the chloroplast. However, these are a minor proportion of the proteases normally encountered in the cytoplasm of eukaryotic cells. Therefore, the chloroplast could potentially be a more sheltered environment for the production of proteins that are particularly susceptible to proteolysis. A major determinant of proteolysis of recombinant proteins is likely to be improper folding and assembly. Many different molecular chaperones, with both eukaryotic and prokaryotic orthologs, have been identified in *C. reinhardtii* chloroplasts. As evidenced by the wide diversity of proteins that have already been expressed in the chloroplast (Table 1), the stromal environment is one that is capable of the proper folding and assembly of heterologous proteins from a large variety of native organisms.

Engineering new proteins and pathways into chloroplasts

Besides the previously discussed production of novel proteins of pharmaceutical interest, algal chloroplasts also have the potential to produce novel metabolites with industrial applications and to accumulate novel compounds with potentially beneficial nutritional value. As an example, there are several opportunities for the exploitation of green algae as biofuel reactors, as exemplified by the recent identification that *C. reinhardtii* can produce hydrogen gas under certain circumstances [45[•]]. The many successes of engineering new metabolic pathways

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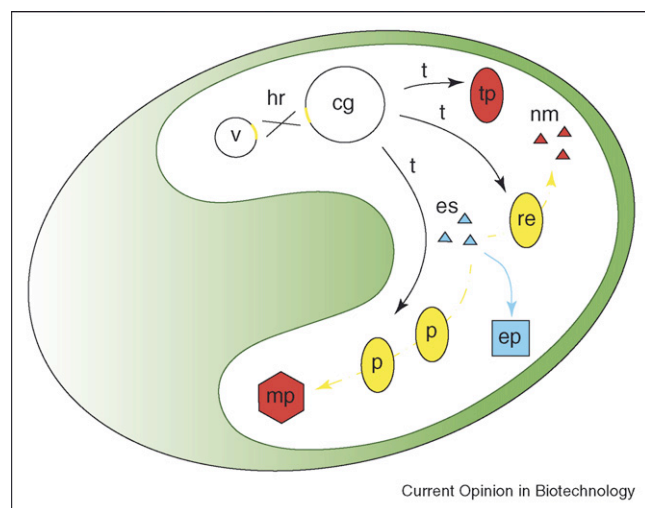
into higher plants chloroplasts [46] suggest the potential of chloroplast engineering in *C. reinhardtii*. With the recent completion of many genome projects, the transfer of metabolic pathways from other organisms to *C. reinhardtii* chloroplasts is now a distinct possibility (e.g. entire vitamin production pathways from higher plants [47]). Likewise, new techniques in genetic manipulation, such as the viable translation of heterologous polycistronic mRNAs in *C. reinhardtii* [48], add new tools for the genetic engineering of the chloroplast.

In addition to engineering recombinant enzymes or pathways that could change metabolic profiles in chloroplasts, novel proteins can also be made. Such potential is found in fusion proteins, in which domains from two different proteins are joined together to make a hybrid protein of novel function. The aforementioned example of a fusion between the FMDV VP1 protein and CTB [36] identifies one such novel fusion protein and supports the possibility of using transgenic chloroplasts of green alga as a mucosal vaccine source. These few examples are just the beginning of what promises to be a rich area of protein engineering, and algal chloroplasts appear to be an ideal platform for this type of protein production.

Conclusions

Researchers have taken advantage of the genetic and molecular techniques available in *C. reinhardtii* to

Figure 2



Engineering *C. reinhardtii* chloroplasts. After integration of the gene via homologous recombination (hr) between the plasmid vector (v) and the chloroplast genome (cg), translation (t) of the recombinant gene can produce a variety of proteins, including recombinant therapeutic proteins (tp), recombinant enzymes (re) that use endogenous substrates (es) and convert them into novel metabolites (nm) instead of endogenous product (ep). Entire pathways of recombinant enzymes (p) can be introduced to produce new metabolic pathways to produce a broader range of metabolic products (mp).

engineer the efficient expression of important recombinant proteins in algal chloroplasts. Even with challenges for improving protein expression and purification lying ahead, rapid and substantial progress has already been made over a relatively short period of time. With the many molecular tools available coupled with the rapid cycling time of *C. reinhardtii*, substantial progress is likely to be made in the near future. As shown in Figure 2, micro-algae are a very attractive system both for the expression of human therapeutic proteins and for the production of specific metabolites, given that algal chloroplasts can assemble and fold complex mammalian proteins. Moreover, they can be grown in containment with relative ease at very large scale in simple facilities that allow for tremendous cost savings. Therapeutic protein expression in *C. reinhardtii* chloroplasts will undoubtedly be optimized further as molecular techniques are advanced, as additional proteins are studied and as *in vivo* and animal trials establish that algal-produced proteins are safe, efficacious, and cost-effective protein therapeutic products.

Update

Recent work from our group has demonstrated robust expression of a bovine serum albumin A3 (M-SAA) protein in the chloroplast of *C. reinhardtii* [52]. This protein, normally found in mammalian colostrum, induces mucin expression in the gut of newborn animals, which in turn acts in prophylaxis of bacterial and viral infections. M-SAA was expressed as 10% TSP by replacement of endogenous *psbA* coding regions with the chloroplast codon-optimized recombinant *m-saa* gene. However, this rendered the strain non-photosynthetic, which would not be appropriate for downstream scale-up processing. Photosynthetic activity was restored to the M-SAA-expressing strain through reintroduction of the *psbA* coding region at an alternate site on the chloroplast genome. Using a chimeric promoter and 5' UTR for the expression of the reintroduced *psbA* gene allowed for continued robust expression of M-SAA in a photosynthetic strain. Cell-based assays employing a human gut epithelial cell line showed that M-SAA produced in and purified from *C. reinhardtii* chloroplasts was bioactive and stimulated mucin production. This type of gut-active protein is an ideal for oral delivery without purification.

Acknowledgements

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