

1 **The algal chloroplast as a synthetic biology platform for production of**  
2 **therapeutic proteins**

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4 Yuliya M. Dyo<sup>1,2</sup> and Saul Purton<sup>3\*</sup>

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6 <sup>1</sup>Molecular Research of Microalgae Laboratory, M. A. Ajtkhozhin Institute of  
7 Molecular Biology and Biochemistry, Almaty, Kazakhstan

8 <sup>2</sup>Department of Biotechnology, Kazakh National Research Technology University,  
9 Almaty, Kazakhstan

10 <sup>3</sup>Algal Research Group, Institute of Structural and Molecular Biology, University  
11 College London, Gower Street, London, WC1E 6BT, United Kingdom

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14 *\*Corresponding author*

15 Saul Purton

16 s.purton@ucl.ac.uk

17 tel. +44 (0)20 76792675

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21

22 **Abbreviations:** GRAS: Generally Recognized As Safe. Synbio: synthetic biology.  
23 CTB: cholera toxin beta-subunit. mAb: monoclonal antibody. TSP: total soluble  
24 protein

25 **Abstract**

26 The chloroplast of *Chlamydomonas reinhardtii* and other microalgae represents an  
27 attractive new platform for the synthesis of recombinant therapeutics using synthetic  
28 biology (synbio) approaches. Transgenes can be designed *in silico*, assembled from  
29 validated DNA parts, and inserted at precise and predetermined locations within the  
30 chloroplast genome to give stable synthesis of a desired recombinant protein.  
31 Numerous recent examples of different therapeutic proteins produced successfully in  
32 the *C. reinhardtii* chloroplast highlight the potential of this green alga as a simple,  
33 low-cost and benign host. Furthermore, features of the alga may offer additional  
34 advantages over more-established microbial, mammalian or plant-based systems.  
35 These include efficient folding and accumulation of the product in the chloroplast; a  
36 lack of contaminating toxins or infectious agents; reduced downstream processing  
37 requirements; the possibility to make complex therapeutics such as immunotoxins,  
38 and the opportunity to use the whole alga as a low-cost oral vaccine. In this article we  
39 review the current status of algal chloroplast engineering with respect to therapeutic  
40 proteins. We also consider future advances in synbio tools, together with  
41 improvements to recipient strains, which will allow the design of bespoke strains with  
42 high levels of productivity.

## 43 **Introduction**

44 Currently, the industrial biotechnology sector is almost exclusively based around the  
45 use of heterotrophic platforms (bacteria, yeasts, mammalian and insect cells) for the  
46 biosynthesis of pharmaceutical proteins, bioactive metabolites or other high-value  
47 products [1]. Nevertheless, the ever-increasing growth of the global bioeconomy and  
48 the need for sustainable alternatives to petrochemical based products is catalyzing  
49 interest in the exploitation of alternative cell factories, including photosynthetic  
50 microalgae and cyanobacteria [2]. Microalgae represent significant untapped potential  
51 for bio-manufacturing because of the extreme biodiversity of the more than 70,000  
52 extant species spread over the eukaryotic tree of life [3, 4]. However, exploitation of  
53 all but a handful of algal species is severely hindered by a paucity of molecular tools  
54 for efficient genetic engineering [2, 5].

55 Of these species, the freshwater chlorophyte *Chlamydomonas reinhardtii* is perhaps  
56 the most advanced microalgal platform, with a suite of molecular tools for both  
57 nuclear and chloroplast transformation, and the on-going development of synthetic  
58 biology strategies for strain engineering [6]. The chloroplast genetic system lends  
59 itself particularly well to synthetic biology since the genome is small (205 kb) and of  
60 low complexity (99 genes) [7], and the precise integration of foreign DNA into any  
61 predetermined loci is readily achieved via homologous recombination [8]. Recently,  
62 there have been a number of reports describing the genetic engineering of the *C.*  
63 *reinhardtii* chloroplast to produce therapeutic proteins, with many shown to be active  
64 and effective in lab-based trials.

65 In this short review we outline the current status and merits of algal chloroplast  
66 transgenics, and survey the different classes of therapeutics being produced for either  
67 human or livestock applications. We also consider the future development of synthetic  
68 biology tools to accelerate the predictive design and creation of bespoke strains. A  
69 more detailed discussion of the history and wider applications of algal chloroplast  
70 engineering is given in several recent reviews [9–12].

71

### 72 **1. The algal chloroplast as a new bio-factory**

73 The chloroplasts of plants and algal cells possess a small polyploid genome (termed  
74 the plastome) derived from the cyanobacterial progenitor of this organelle. The algal

75 plastome is composed of ~100–200 genes, most of which encode core components of  
76 the photosynthetic complexes and the chloroplast's transcription-translation apparatus.  
77 The genetic system reflects its bacterial ancestry and is essentially prokaryotic in  
78 nature, with a eubacterial-like RNA polymerase and 70S ribosome, and many genes  
79 arranged into co-transcribed units [13]. However, introns are present in some  
80 chloroplast genes, and the regulation of gene expression occurs largely at post-  
81 transcriptional steps (rather than at the transcriptional level) with numerous nuclear-  
82 encoded protein factors imported into the chloroplast to mediate RNA processing,  
83 splicing and stabilization, and translation initiation [14].

84 DNA transformation of the chloroplast was first reported in 1988 using the single-  
85 celled green alga, *Chlamydomonas reinhardtii* [15]. Since that time the tools and  
86 techniques for chloroplast genetic engineering of *C. reinhardtii* have advanced  
87 significantly [8, 16]. More recently, chloroplast transformation has been achieved for  
88 other microalgal species including the green algae *Haematococcus pluvialis* and  
89 *Dunaliella tertiolecta* [17, 18], the red alga *Cyanidioschyzon merolae* [19], and the  
90 diatom *Phaeodactylum tricorutum* [20]. However, progress to-date in the  
91 development of the algal chloroplast as a platform has almost exclusively focused on  
92 *C. reinhardtii* with over 100 reports in the literature of production of recombinant  
93 proteins in this species. Chloroplast transformation is also feasible for a number of  
94 plant species, with advanced genetic engineering technologies available for tobacco  
95 (*Nicotiana tabacum*) and several other plants such as tomato, potato and petunia [21].  
96 Although plant chloroplasts represent an attractive low-cost and easily scalable  
97 platform for synthesis of biopharmaceuticals [22], there are fundamental challenges  
98 associated with the use of crop plants for drug production. These include the  
99 difficulties of ensuring rigorous good manufacturing practice during glasshouse or  
100 field cultivation, and concerns of escape and contamination of food crops [23]. In  
101 contrast, a microalgal platform circumvents many of these issues since these  
102 microorganisms can be grown under tightly controlled, sterile and contained  
103 conditions in closed fermenter or photobioreactor systems. Furthermore, several  
104 microalgal species including *C. reinhardtii* have GRAS (Generally Recognized As  
105 Safe) status and are therefore considered free of harmful viral, prion or endotoxin  
106 contaminants, thereby simplifying procedures for product purification. The safety of  
107 these species also offers the possibility of topical application of a biopharmaceutical

108 such as an anti-microbial protein using a crude cell lysate of the alga (e.g. formulated  
109 into a spray or cream), which would avoid costly investment in purification.  
110 Alternatively, it might be possible to use the whole alga for oral delivery (to animals,  
111 if not to humans) of vaccines, enzymes, or hormones – with the dried cells exploited  
112 as a natural method of encapsulation and storage at room temperature that overcomes  
113 the need for a cold chain [24].

114 Typically, transgenic DNA is introduced into the chloroplast by bombardment of an  
115 algal lawn or plant tissue with DNA-coated gold microparticles. Alternative DNA  
116 delivery strategies include electroporation [25] or agitation of a DNA/cell suspension  
117 in the presence of glass beads [26]. DNA integration into the plastome occurs almost  
118 exclusively via homologous recombination between matching sequence on the  
119 incoming DNA and plastome sequence [8]. Consequently, transgenes can be precisely  
120 targeted to any locus by flanking the DNA with chloroplast sequences upstream and  
121 downstream of the target locus as shown in Figure 1. Several selection strategies have  
122 been developed based on the use of bacterial antibiotic-resistance genes such as *aadA*  
123 and *aphA6* [8], however a superior selection strategy involves the rescue of a  
124 chloroplast mutant to phototrophy. As illustrated in Figure 1, this results in marker-  
125 free transformants in which the only foreign DNA in the plastome is the gene-of-  
126 interest [27, 28]. Expression of the gene is achieved by fusing the coding sequence to  
127 promoters and untranslated regions from highly expressed endogenous genes such as  
128 the photosynthesis genes *psaA* and *psbA*. The efficiency of translation can be  
129 significantly improved by using synthetic coding sequence that is optimized to match  
130 the AT-rich codon bias seen in chloroplast genes [9]. Additionally, biocontainment  
131 can be built into the transgene by replacing several tryptophan codons (UGG) with the  
132 UGA stop codon and using an orthogonal tryptophan tRNA to recognize these  
133 internal stop codons in the chloroplast [29]. Although almost all transgenes inserted  
134 into the *C. reinhardtii* chloroplast to-date have been constitutively expressed,  
135 regulation of transgene expression can be achieved using a vitamin-based system.  
136 Here, the expression of a nuclear gene encoding a factor essential for translation of  
137 the chloroplast *psbD* gene is repressed by addition of vitamin B<sub>12</sub> and thiamine to the  
138 medium. Any transgene fused to the *psbD* 5'UTR is therefore translated only in the  
139 absence of the vitamins [30].

140 Using these molecular tools, over 100 different recombinant proteins have been  
141 successfully produced in the algal chloroplast. Reported yields are generally in the  
142 range of 0.1% to 5% total soluble protein (TSP), although caution should be exercised  
143 when making comparisons since the preparation of soluble extracts and the assay used  
144 for quantification differs between groups. A better measure is perhaps protein yield  
145 per gram of dried biomass [31]. Whilst such levels are below that of established  
146 recombinant platforms, new synthetic biology approaches (see below) and molecular-  
147 genetics strategies based on our understanding of chloroplast gene regulation in *C.*  
148 *reinhardtii* are now leading to significant improvements in yield.

149

## 150 **2. Biopharmaceuticals made in the *C. reinhardtii* chloroplast**

151 A review of the literature identifies over 40 different therapeutic proteins successfully  
152 produced in *C. reinhardtii* chloroplast with many shown to be bioactive, as  
153 summarized in Table 1. In most cases these are single subunit proteins and therefore  
154 involve the introduction of only a single transgene, although there have been a few  
155 examples of multigenic engineering of the plastome [32, 33]. To-date all the  
156 therapeutic proteins reported are soluble and accumulate in the chloroplast stroma,  
157 save for a single report of targeting of an antibody fragment to the thylakoid lumen  
158 [34] although membrane-anchored proteins have been successfully produced in the  
159 algal chloroplast [35].

### 160 **2.1 Subunit vaccines**

161 Edible microalgae such as *C. reinhardtii* are attractive systems for oral delivery of  
162 protein vaccines. This is especially the case for farmed animals such as fish and  
163 poultry where alternative vaccination strategies such as injection of a purified vaccine  
164 are impractical or prohibitively expensive given the small size and low value of the  
165 individual animal. As detailed in Table 1, antigens from viral, bacterial and malarial  
166 parasite pathogens have been produced in the algal chloroplast, and in many of these  
167 cases an immunogenic response in model animals has been demonstrated. In several  
168 studies, a protein adjuvant (cholera toxin B subunit: CTB) has been fused to the N-  
169 terminus of the antigen. CTB assembles into a pentameric structure and acts as an  
170 effective mucosal adjuvant by binding GM1 ganglioside receptors on gut epithelial  
171 cells. For those vaccines aimed at the aquaculture, poultry and livestock industries,

172 the whole dried algae could be formulated into the animal feed. For the malarial  
173 vaccines, the need for very low cost and simple production technologies for any  
174 treatment in developing countries may ultimately overcome the current strict  
175 regulations for vaccine purification and lead to the use of such whole cell preparations  
176 as oral therapeutics [63]. Importantly, several studies have shown that the chloroplast-  
177 produced vaccines in lyophilized algae remain stable and active at room temperature  
178 over extended periods. For example, Dreesen et al. [40] showed that their CTB-D2  
179 vaccine was stable for more than 1.5 years at room temperature, and Gregory et al.  
180 [43] showed that their CTB-Pfs25 vaccine remained active for over six months at  
181 22°C (although activity was reduced at 37°C). The lack of a requirement for a cold-  
182 chain would obviously reduce the complexity and cost of vaccine distribution. Drying  
183 the algae also serves to bio-encapsulate the vaccine within multiple layers (the double  
184 membrane of the chloroplast, the cell membrane and the cell wall), thereby helping to  
185 protect the vaccine from oxidation during storage, and degradation within the animal  
186 stomach during delivery to the gut epithelium. Furthermore, it is possible that the  
187 components of the algal cell wall could act as an effective mucosal adjuvant [64].

## 188 **2.2 Antibodies and immunotoxins**

189 Complex proteins such as monoclonal antibodies (mAb) that contain multiple  
190 disulphide bonds are difficult to produce in prokaryotes and therefore have to be made  
191 using eukaryotic platforms [65]. Currently, almost all marketed antibodies are  
192 produced in mammalian cell culture, and are therefore expensive and limited in  
193 availability [66]. Although the chloroplast does not possess the machinery for  
194 glycosylation of proteins, work by the group of Mayfield has shown that the algal  
195 chloroplast is capable of correctly folding and assembling aglycosylated antibodies  
196 that are able to bind their target. An early study [52] produced a mAb against  
197 glycoprotein D of the herpes simplex virus (HSV) as a large single chain in which the  
198 variable region of the light chain was fused via a linker to the IgA heavy chain. This  
199 protein accumulated as a soluble protein that could form a dimer linked by disulphide  
200 bonds and was shown to bind the HSV glycoprotein *in vitro*. Subsequently, Tran et al.  
201 [33] demonstrated that a mAb comprising separate heavy and light chains that were  
202 co-expressed in the chloroplast assembled correctly into a functional tetramer of two  
203 heavy chains and two light chains held together by multiple disulphide bonds. The  
204 mAb was directed against the PA83 antigen of *Bacillus anthracis* and the study

205 showed that the chloroplast-produced mAb bound to the antigen with a similar  
206 affinity to a mAb produced in a mammalian system.

207 The Mayfield group have extended their studies to show that immunotoxins – fusion  
208 proteins comprising antibodies linked to cytotoxic proteins which have applications in  
209 cancer treatment – can also be produced in the algal chloroplast. Production of such  
210 cytotoxic proteins in eukaryotic hosts such as CHO cells or yeast is not feasible  
211 because of the lethal effect of the toxin on the cytosolic translation apparatus,  
212 whereas production in prokaryotic systems is challenging because of the  
213 difficulty of folding and assembling such complex molecules. In two impressive  
214 papers, the group achieved the synthesis of immunotoxins comprising a single  
215 chain antibody recognizing the CD22 surface receptor from B-cells fused either to  
216 domain II and III of Exotoxin A from *Pseudomonas aeruginosa* [54] or to the  
217 ribosome inactivating protein, gelonin, from *Gelonium multiflorm* [55]. Both  
218 immunotoxins were capable of specifically binding B-cells *in vitro* and that in the  
219 case of the immunotoxin Exotoxin A, survival of mice implanted with a human B-cell  
220 tumor, the life-span was extended. This work showed that the algal chloroplast not  
221 only possesses the machinery necessary to fold and assemble complex eukaryotic  
222 proteins, but that the 70S ribosomes are unaffected by the toxic proteins and the  
223 organelle is able to completely contain the protein preventing any inhibitory effect on  
224 the host's cytosolic ribosomes. The chloroplast therefore presents an attractive sub-  
225 cellular compartment for efficient production of these highly complex therapeutics.

### 226 **2.3 Other therapeutic proteins**

227 As detailed in Table 1, numerous other classes of therapeutic proteins have been  
228 successfully produced in the *C. reinhardtii* chloroplast and shown to be biologically  
229 active. These include hormones such as human growth hormone [28], anti-  
230 hypertensive peptides [62], cancer therapeutics [61], antibody mimics [56],  
231 autoantigens [49], wound healing factors [56] and anti-bacterial enzymes [31]. These  
232 examples serve to illustrate the potential of the chloroplast as a platform for a wide  
233 variety of recombinants. However, two areas where the GRAS benefits of microalgae  
234 such as *C. reinhardtii* could be particularly exploited is in allergen-specific  
235 immunotherapy (AIT) and in the delivery of gut-active proteins to livestock.  
236 Treatment of food allergies such as peanut allergy using AIT delivered via oral,  
237 sublingual or epicutaneous routes is a promising strategy. However, the high risk of



238 adverse side effects from the complex protein mix in peanut extracts means that  
239 immunotherapy using such extracts is not recommended in clinical practice. However,  
240 recombinant allergens can be purified without concern for contamination by cross-  
241 reactive peanut proteins, and are therefore an attractive alternative to native allergens  
242 for immunotherapy and allergy diagnostics [67]. Furthermore, the recombinant  
243 proteins can be modified to reduce the severity of the allergic response. Gregory et al.  
244 [50] showed that major peanut allergens produced in *C. reinhardtii* conferred  
245 protection from peanut-triggered anaphylaxis in a mouse model. This study hopefully  
246 will pave the way for human trials of AIT using oral delivery of the recombinant  
247 algae.

248 Mammary-associated serum amyloid (M-SAA) is a component of mammalian  
249 colostrum and induces mucin synthesis in gut epithelial cells, resulting in increased  
250 protection of newborns against bacterial infections in the intestine [68]. Algal-  
251 produced M-SAA provided in the feed could provide this protective agent for  
252 newborn mammals that lack a source of colostrum, serving as a prophylactic against  
253 infection. Manuell et al. [57] showed that M-SAA produced in *C. reinhardtii* was able  
254 to stimulate mucin production in human gut epithelial cell lines. Another feed additive  
255 that has significant health and economic benefits in agriculture is phytase. In plant-  
256 derived animal feed, nearly 80% of the total phosphorus content is stored as phytate.  
257 However, phytate is poorly digested by monogastric animals such as swine, poultry  
258 and fish, as they lack the hydrolytic enzyme phytase. In addition, phytate also chelates  
259 important dietary minerals and essential amino acids. Therefore, dietary  
260 supplementation with bioavailable phosphate and exogenous phytases are required to  
261 achieve optimal animal growth [69]. Two separate studies have produced recombinant  
262 phytases in *C. reinhardtii* [58, 60] with the earlier study demonstrating that dried algal  
263 biomass fed to broiler chicks significantly reduced phytate excretion, and the latter  
264 study calculating that costs of the production in microalgae are comparable to  
265 commercial supplies of phytase. It is possible to envisage further cost savings in, for  
266 example, pig feed by ‘pyramiding’ different gut-active proteins such that a single alga  
267 produces multiple recombinant products such as phytase, M-SAA, vaccines and anti-  
268 bacterials.

269

270 **4. Emerging synthetic biology approaches**

271 Currently, most recombinant expression in the algal chloroplast involves single gene  
272 constructs created using conventional restriction enzyme-based cloning approaches.  
273 This limits the rate at which new transgenic lines can be produced and tested, and in  
274 particular, how many different permutations of constructs (different promoters,  
275 coding variants, regulatory elements, *etc.*) can be evaluated. We are now starting to  
276 see the application of synthetic biology principles to plastome engineering with the  
277 adoption of assembly standards such as Golden Gate and the creation of libraries of  
278 validated DNA parts that allow rapid one-step assembly of all the parts [27, 70, 71].  
279 In the near future, we may see much more ambitious design strategies that involve  
280 extensive re-design of the plastome *in silico* such that large tracts of non-essential  
281 DNA are removed [72], essential endogenous genes are refactored into functional  
282 clusters [73] and multiple transgenes are engineered into different loci. Assembly and  
283 delivery of such synthetic genomes is technically feasible, as shown by O'Neill et al.  
284 [74] who demonstrated that the entire *C. reinhardtii* plastome could be assembled in  
285 yeast and transformed into *C. reinhardtii* by microparticle bombardment. The  
286 challenge is to develop selection strategies that allow the clean replacement of the  
287 endogenous plastome with the synthetic version without undesirable recombination  
288 events between the two resulting in the creation of chimeric plastomes [74].

289 Another challenge is to improve significantly the product yield through the use of  
290 synthetic *cis* elements to drive expression. Currently, the promoter and 5'UTR used to  
291 express transgenes are derived from endogenous photosynthetic genes. In some cases,  
292 expression levels can be improved by using the stronger promoter from the gene for  
293 the 16S ribosomal RNA fused to the 5'UTR of a photosynthetic gene [27, 75].  
294 However, more often it is the performance of the 5'UTR that is the bottleneck [76],  
295 with the efficiency of translation constrained either by the same feedback regulation  
296 that prevents over-accumulation of individual photosynthetic subunits in the absence  
297 of their assembly partners (so called 'Control by Epistasy of Synthesis'), or by  
298 competition with the corresponding endogenous gene transcript for *trans*-acting  
299 factors that are required for transcript stability or translation, but are present in  
300 limiting concentration in the chloroplast [77]. Strategies to overcome this involve  
301 either replacement of the 5'UTR of the endogenous gene with that from another  
302 photosynthetic gene [78], or more elegantly to develop synthetic variants of the  
303 5'UTR that are no longer subject to these limitations and therefore give improved

304 expression of the transgene [79]. Further studies into the design of synthetic  
305 promoters and UTRs, combined with improved knowledge of codon optimization  
306 rules, will advance the average recombinant protein yield from the current value of  
307 ~1% TSP to the >10% level required of a commercial platform.

308

### 309 **5. Summary and perspectives**

310 The microalgal chloroplast has clear potential as a novel industrial production  
311 platform for biopharmaceuticals. The continued development of synthetic biology  
312 tools for chloroplast engineering of *C. reinhardtii* will strengthen this potential by  
313 accelerating the creation of designer transgenic lines yielding high levels of the target  
314 protein. However, this increase in yield needs to be coupled with improvements in  
315 phototrophic algal biomass production in order to make the platform commercially  
316 competitive. Such improvements will come from a combination of media  
317 optimization [80], improvements in photobioreactor (PBR) design [81], and strain  
318 domestication such as selection for reduced light-antenna mutants that show higher  
319 productivity in PBRs as a consequence of greater light penetration [82]. Alternatively,  
320 it might prove more cost effective to switch to mixotrophic production in PBRs, or  
321 heterotrophic cultivation in fermenters where much high biomass productivity can be  
322 achieved [83]. In the case of *C. reinhardtii* acetate is used as the fixed carbon source,  
323 although strain engineering could enable the alga to be cultivated using glucose or  
324 sucrose as the carbon source [84]. Finally, the development of chloroplast  
325 transformation technology for other GRAS species such as *Dunaliella salina*,  
326 *Chlorella vulgaris* and *Haematococcus pluvialis* that are already grown commercially  
327 will provide opportunities for larger-scale and lower cost production of therapeutic  
328 proteins in algae.

329

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336

337 **Conflicts of interest**

338 The authors declare that there are no conflicts of interest.

339

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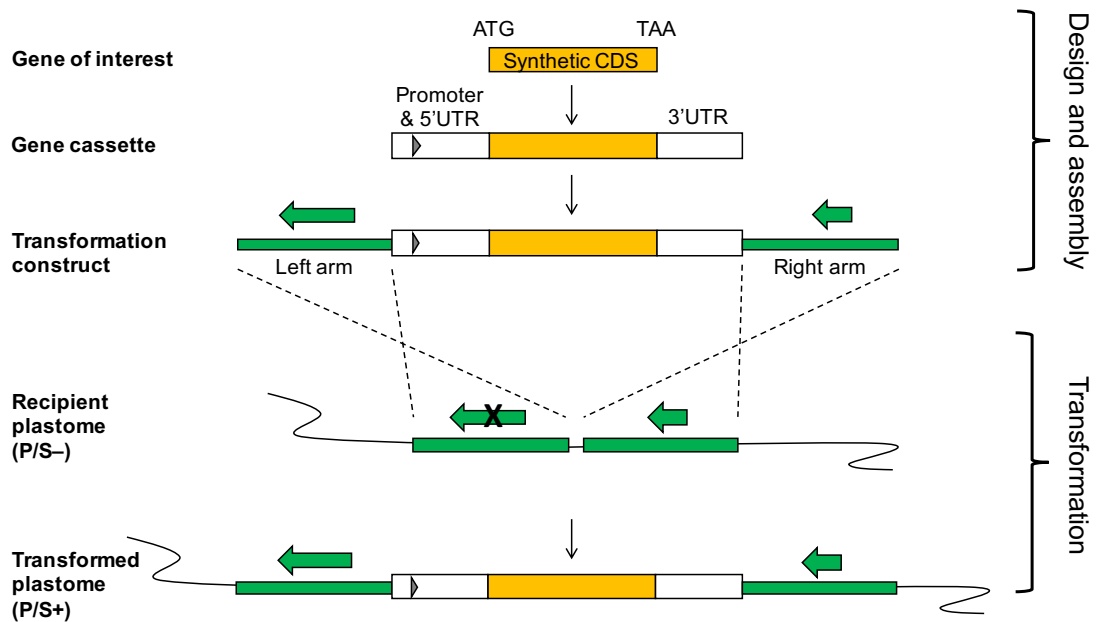
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628 **Figure 1:** Marker-free strategy for introducing transgenes into the *C. reinhardtii*  
629 chloroplast. The gene-of-interest (GOI) is codon-optimised to match chloroplast genes,  
630 and assembled into a transformation construct using a ‘one-step’ method such as  
631 Golden Gate or Gibson assembly. The left and right arms are chloroplast DNA parts  
632 that ensure insertion into a specific intergenic region via homologous recombination  
633 between the arms and the recipient plastome. One of the arms carries a wild-type copy  
634 of a gene that is essential for photosynthesis, and selection is based on the repair of a  
635 mutated form of this gene (indicated with an ‘X’) in the photosynthesis-deficient  
636 (P/S-) recipient strain. The resulting transformant is therefore restored to phototrophy  
637 (P/S+) with only the GOI introduced into the plastome.

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640 **Table 1:** Therapeutic proteins produced in the *C. reinhardtii* chloroplast.

protein name	description	key findings	yield <sup>1</sup>	reference
<i>Subunit vaccines</i>				
CTB-VP1	CTB adjuvant fused to structural protein VP1 of Foot-and-Mouth Disease Virus, a pathogen of livestock.	First report of a potential mucosal vaccine produced in algae.	3% TSP	[36]
E2	Structural protein E2 of classical swine fever virus, a pathogen of swine.	Elicited a strong immunogenic response in mice following subcutaneous, but not oral, administration.	1.5 – 2% TSP	[37]
p57	Protein p57 from <i>Renibacterium salmoninarum</i> , the causative agent of bacterial kidney disease in salmonid fish.	Both live and freeze-dried algae elicited an immunogenic response when fed to fish.	nd	[38]
VP28	Envelope protein VP28 of White Spot Syndrome Virus, a pathogen of crustaceans.	Codon optimization of the VP28 gene, and strain context appeared to have a marked effect on protein accumulation.	>20% TCP	[39]
CTB-D2	CTB adjuvant fused to D2 fibronectin-binding domain of bacterial pathogen, <i>Staphylococcus aureus</i> .	Oral delivery to mice of dried algae elicited specific mucosal and systemic immune responses and protected the mice against infection.	0.7% TSP	[40]
AcrV and VapA	Antigens from <i>Aeromonas salmonicida</i> , a bacterial pathogen	Choice of promoter/5'UTR and host strain significantly improved expression levels.	0.8% TSP (AcrV) 0.3% TSP (VapA)	[41]

	of salmonids.			
Pfs25 and Pfs28	Surface protein antigens from malarial parasite, <i>Plasmodium falciparum</i> .	Recombinant antigens shown to be structurally similar to the native proteins. Antibodies to Pfs25 bound the sexual stage parasite and exhibited transmission blocking activity.	0.5% TSP (Pfs25) 0.2% TSP (Pfs28)	[42]
CTB-Pfs25	CTB adjuvant fused to Pfs25 surface antigen of <i>P. falciparum</i>	Oral delivery to mice of the dried algae elicited an immune response.	0.09% TSP	[43]
Pfs48/45	Surface protein antigen from <i>P. falciparum</i> .	The recombinant antigen shown to accumulate in the chloroplast in the correct structural conformation.	n.d.	[44]
E7GGG	A mutated, attenuated form of the E7 oncoprotein from Human Papilloma Virus type 16	Induction of anti-E7 IgGs, and E7-specific T-cell proliferation detected in mice following sub-cutaneous injection of total algal extract. High levels of tumor protection obtained following challenge with a tumor cell line expressing the E7 protein.	0.12% TSP	[45]
E7GGG-AadA	E7GGG fused to the bacterial spectinomycin resistance enzyme, AadA.	Subcutaneous injection of algal extracts into mice showed high production of E7-specific antibodies, but low activation of E7-specific CD8+ cells.	n.d.	[46]
MPT64	Secreted antigen of <i>Mycobacterium tuberculosis</i> .	High-level expression obtained using the 16S rRNA promoter fused to the <i>atpA</i> 5'UTR.	n.d.	[27]

HA	Hemagglutinin of Avian Influenza Virus H5	Ocular administration of the recombinant HA to broiler chickens resulted in an immunogenic response.	770 µg/g DB	[47]
CTB-p210	CTB adjuvant fused to the p210 epitope of ApoB100, the main apolipoprotein in low density lipoproteins associated with atherosclerosis.	Oral delivery of fresh algae to mice elicited an immune response.	60 µg/g FB	[48]
<b><i>Autoantigens</i></b>				
hGAD65	Human glutamic acid decarboxylase	Purified hGAD65 shown to be immunoreactive to diabetic sera and able to induce proliferation of spleen cells in a diabetic mouse model.	0.25–0.3% TSP	[49]
hIL4	Human Interleukin 4	First report of algal transplastomic lines produced by electroporation.	n.d.	[25]
<b><i>Allergens</i></b>				
Ara h 1 core domain and Ara h 2	Major peanut allergens	Recombinant protein conferred protection from peanut-triggered anaphylaxis in mouse model.	n.d.	[50]
Bet v 1	Major birch pollen allergen	The algal-derived Bet v 1 had similar	0.01–0.04% TSP	[51]



		immunologic properties to its <i>E. coli</i> - produced counterpart.		
<b>Monoclonal antibodies (mAb)</b>				
HSV8-lsc	Large single-chain (lsc) antibody against glycoprotein D of herpes simplex virus.	First demonstration of accumulation of soluble, correctly folded lsc antibody in the algal chloroplast including dimer formation via inter-molecular disulfide bonds.	0.5 % TSP	[52]
83K7C	Human IgG1 antibody against anthrax protective antigen 83.	First demonstration that heavy and light chains synthesized in the same chloroplast assemble into a full-length functional mAb.	~100 µg/g DB	[33]
<b>Nanobodies</b>				
V <sub>H</sub> H	Variable domain of camelid heavy chain-only antibodies targeting botulinum neurotoxin	V <sub>H</sub> H proteins were shown to bind with high affinity to the toxin, and to survive in the gut of mice fed fresh whole algae.	1.4 – 4.6% TSP	[53]
<b>Immunotoxins</b>				
αCD22CH23PE40	Chimeric antibody to B-cell surface antigen CD22 fused to the enzymatic domain of exotoxin A from <i>Pseudomonas aeruginosa</i> .	Immunotoxin was soluble and able to form a dimeric structure. It was able to kill B cells <i>in vitro</i> and significantly prolonged the survival of mice with implanted B-cell tumours.	0.2–0.3% TSP	[54]
αCD22CH23Gel	Chimeric antibody to B-cell surface	As above, the immunotoxin formed a dimer	0.1–0.2% TSP	[55]

	antigen CD22 fused to 80S ribosome inactivating protein gelonin from <i>Gelonium multiflorum</i> .	and was capable of binding to, and reducing the viability of, B-cell lymphomas.		
<b><i>Antibody mimics</i></b>				
10FN3	Tenth binding domain of human fibronectin type III.	Low yield of 10FN3 significantly improved by expression as a SAA-10FN3 fusion.	n.d.	[56]
14FN3	Fourteenth binding domain of human fibronectin type III.	Purified as a soluble protein of the expected molecular mass.	3%	[56]
<b><i>Growth factors</i></b>				
VEGF	Vascular endothelial growth factor	Bioactivity of purified protein confirmed in VEGF receptor binding assays.	2% TSP	[56]
hGH	Human growth hormone	Algal cell lysate showed hGH bioactivity in mammalian cell proliferation assay.	0.5 mg/L culture	[28]
<b><i>Gut-active proteins</i></b>				
M-SAA	Bovine mammary-associated serum amyloid	Purified M-SAA stimulated mucin production in human gut epithelial cell lines.	>5% TSP	[57]
AppA	Phytase from <i>Escherichia coli</i>	Dried algal biomass fed to broiler chicks significantly reduced phytate excretion.	n.d.	[58]

NCQ	Chimeric protein comprising 20 known bioactive peptide sequences from milk proteins	The artificial protein accumulated to readily detectable levels in algal lines.	0.16 – 2.4% TSP	[59]
PhyA-E228K	Phytase from <i>Aspergillus niger</i>	Algal cell lysate showed high phytase activity <i>in vitro</i> at optimal pH of 3.5.	n.d.	[60]
<b><i>Wound healing factors</i></b>				
HMGB1	High mobility group protein B1	Purified protein showed similar bioactivity to commercial HMGB1 produced in bacteria.	2.5% TSP	[56]
<b><i>Anti-bacterials</i></b>				
Cpl-1 and Pal	Endolysins from bacteriophage of <i>Streptococcus pneumoniae</i> .	Algal cell lysates and purified endolysins showed effective anti-bacterial activity against various serotypes of <i>S. pneumoniae</i> .	0.9–1.2% TSP ~1.3 mg/g DB	[31]
<b><i>Cancer cell therapeutics</i></b>				
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand	Soluble protein accumulated in the chloroplast.	0.43%–0.67% TSP	[61]
<b><i>Anti-hypertensive peptides</i></b>				

VLPLP	Chimeric protein containing anti-hypertensive peptides	Intragastric administration of the dried algae to a rat model significantly reduced systolic blood pressure.	0.292 mg/g DB	[62]
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