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

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

Abstract

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

Introduction

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

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

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

1. The algal chloroplast as a new bio-factory

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

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

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

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

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

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

2. Biopharmaceuticals made in the C. reinhardtii chloroplast

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

2.1 Subunit vaccines

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

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

2.2 Antibodies and immunotoxins

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

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

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

2.3 Other therapeutic proteins

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

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

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

4. Emerging synthetic biology approaches

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

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

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

5. Summary and perspectives

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

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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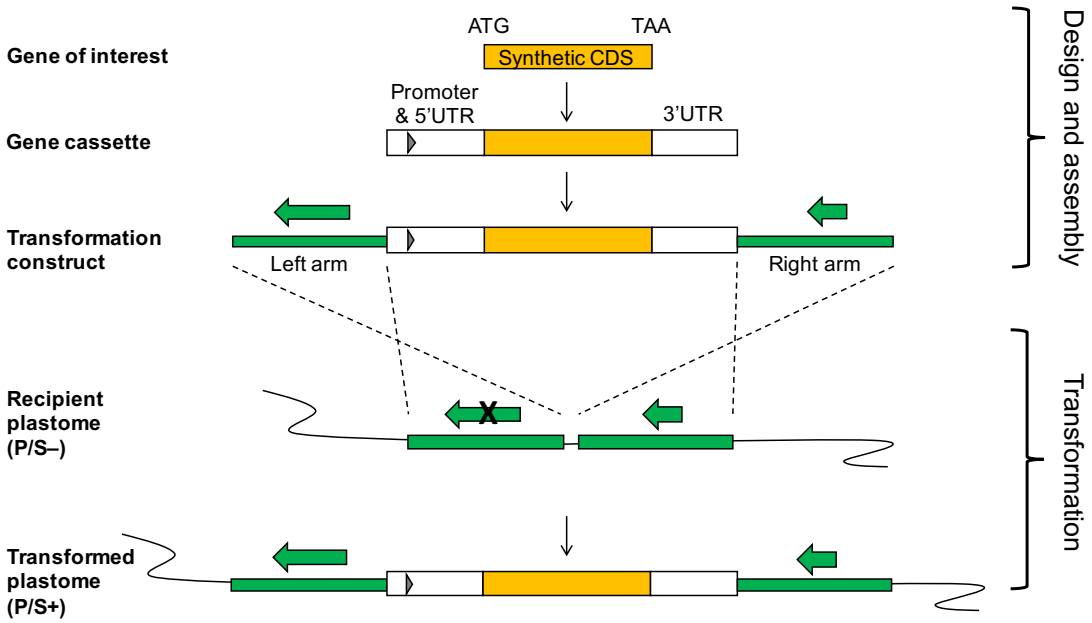

Figure 1: Marker-free strategy for introducing transgenes into the *C. reinhardtii* chloroplast. The gene-of-interest (GOI) is codon-optimised to match chloroplast genes, and assembled into a transformation construct using a 'one-step' method such as Golden Gate or Gibson assembly. The left and right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

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

protein name	description	key findings	yield[1]	reference
Subunit vaccines				
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 *Renibacterium salmoninarum*, 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, *Staphylococcus aureus*.	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 *Aeromonas salmonicida*, 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, *Plasmodium falciparum.*	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 *P. falciparum*	Oral delivery to mice of the dried algae elicited an immune response.	0.09% TSP	[43]
Pfs48/45	Surface protein antigen from *P. falciparum.*	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 *Mycobacterium tuberculosis*.	High-level expression obtained using the 16S rRNA promoter fused to the *atpA* 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]
Autoantigens				
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]
Allergens				
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 *E. coli* - produced counterpart.		
Monoclonal antibodies (mAb)				
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]
Nanobodies				
V_HH	Variable domain of camelid heavy chain-only antibodies targeting botulinum neurotoxin	V_HH 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]
Immunotoxins				
αCD22CH23PE40	Chimeric antibody to B-cell surface antigen CD22 fused to the enzymatic domain of exotoxin A from *Pseudomonas aeruginosa.*	Immunotoxin was soluble and able to forms a dimeric structure. It was able to kill B cells *in vitro* 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 *Gelonium multiflorum.*	and was capable of binding to, and reducing the viability of, B-cell lymphomas.		
Antibody mimics				
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]
Growth factors				
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]
Gut-active proteins				
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 *Escherichia coli*	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 *Aspergillus niger*	Algal cell lysate showed high phytase activity *in vitro* at optimal pH of 3.5.	n.d.	[60]
Wound healing factors				
HMGB1	High mobility group protein B1	Purified protein showed similar bioactivity to commercial HMGB1 produced in bacteria.	2.5% TSP	[56]
Anti-bacterials				
Cpl-1 and Pal	Endolysins from bacteriophage of *Streptococcus pneumoniae.*	Algal cell lysates and purified endolysins showed effective anti-bacterial activity against various serotypes of S. *pneumoniae.*	0.9–1.2% TSP ~1.3 mg/g DB	[31]
Cancer cell therapeutics				
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand	Soluble protein accumulated in the chloroplast.	0.43%–0.67% TSP	[61]
Anti-hypertensive peptides				

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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