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Feature Review

The Engineered Chloroplast Genome Just Got Smarter

Shuangxia Jin[1,2] and Henry Daniell[1,*]

Chloroplasts are known to sustain life on earth by providing food, fuel, and oxygen through the process of photosynthesis. However, the chloroplast genome has also been smartly engineered to confer valuable agronomic traits and/or serve as bioreactors for the production of industrial enzymes, biopharmaceuticals, bioproducts, or vaccines. The recent breakthrough in hyperexpression of biopharmaceuticals in edible leaves has facilitated progression to clinical studies by major pharmaceutical companies. This review critically evaluates progress in developing new tools to enhance or simplify expression of targeted genes in chloroplasts. These tools hold the promise to further the development of novel fuels and products, enhance the photosynthetic process, and increase our understanding of retrograde signaling and cellular processes.

Two Decades of Chloroplast Genetic Engineering

Almost two decades ago, the tobacco (*Nicotiana tabacum*) chloroplast genome was engineered to confer herbicide and insect resistance, outperforming nuclear transgene expression by several hundred-fold [1,2]. Another milestone was engineering salt-tolerance in carrot (*Daucus carota*), a species requiring somatic embryogenesis [3]. This was followed by several other reports utilizing somatic embryogenesis including cotton (*Gossypium hirsuturm*) [4] and soybean (*Glycine max*) [5,6]. Today, several edible crops have been transformed utilizing organogenesis, including lettuce (*Lactuca sativa*), cabbage (*Brassica oleraceavar*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), and sugar beet (*Beta vulgaris*) [7–11]. However, plastid transformation of cereal crops remains elusive.

Current chloroplast genome-engineering projects have led to stable integration and expression of transgenes from different kingdoms including bacterial, viral, fungal, animal, and human genes to express biopharmaceutical proteins, antibiotics, vaccine antigens, industrial enzymes, and biomaterials to confer valuable agronomic traits. High levels of expression, multigene engineering in a single transformation event, transgene containment via maternal inheritance, and minimal pleiotropic effects due to subcellular compartmentalization of toxic transgene products are typical advantages of transforming the chloroplast over the nuclear genome. In this review we critically evaluate recent advances in this expanding field. Biopharmaceuticals expressed in chloroplasts have advanced to clinical studies by the pharmaceutical industry, providing a clear indication of the current maturity of this field and the importance of this approach. Hyperexpression of biopharmaceuticals in healthy plants (making up to 70% of total leaf protein) and the ability to express in edible leaves permits oral delivery and significantly reduces production costs. Several metabolic and genetic diseases including Alzheimer's, diabetes, hypertension, hemophilia, and retinal diseases have been successfully treated with therapeutic proteins made in chloroplasts. Several new tools including Gateway/modular chloroplast vectors, splicing exons to facilitate expression of eukaryotic genes using overlapping PCR primers, multigene engineering concepts, and the application of species-specific vectors have significantly improved the efficiency of chloroplast genome engineering. RNA interference has been explored

Trends

Hyperexpression of biopharmaceuticals in edible leaf chloroplasts documents a recent breakthrough in low-cost oral delivery of biopharmaceuticals that are bio-encapsulated in plant cells. This will enable treating human metabolic or genetic diseases, such as Alzheimer's, diabetes, hypertension, hemophilia, and retinal diseases.

New tools for smart chloroplast genome engineering are now available, including Gateway/modular vectors, RNAi interference, and species-specific vectors for efficient transformation of new crop chloroplast genomes and enhanced transgene expression.

Multigene metabolic engineering of chloroplasts can be used to produce high value bioproducts.

Single chloroplast transgenes are used to confer biotic/abiotic stress tolerance or enhance biomass.

Regulation of the nuclear genome is enabled by genes expressed in chloroplasts via retrograde signaling.

[1]Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA
[2]National Key Laboratory of Crop Genetic Improvement, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China

*Correspondence: hdaniell@upenn.edu (H. Daniell).

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for the first time via the chloroplast genome to silence genes in the insect gut. Single genes conferring diverse agronomic traits including enhanced biomass and resistance to biotic/abiotic stress have been explored. Most importantly, transgenes engineered via the chloroplast genome can regulate nuclear gene expression, offering a valuable tool to understand **retrograde signaling** and other cellular processes.

The Art of Chloroplast Genome Engineering – Evolving New Concepts

Chloroplast transformation requires double homologous recombination (Figure 1A) [12,13]. Therefore, two chloroplast DNA segments are used as flanking sequences in chloroplast vectors to insert the transgene cassette into an intergenic spacer region, without disrupting any functional genes. The first debate in this field was to find the ideal site for transgene integration. Two opposing theories emerged: insertion of transgenes into transcriptionally-silent spacer regions (in which chloroplast genes are located on opposite DNA strands and in opposite orientations – the Maliga concept) or insertion into transcriptionally-active spacer regions (within chloroplast operons – the Daniell concept). The advantages of each site were tested recently by insertion of the *lux* operon with an identical expression cassette at both sites; the transcriptionally-active spacer region was found to offer a 25-fold higher level of expression [14], and authors attributed this to higher read-through transcriptional activity. To date, one of the most commonly used site of transgene integration is the transcriptionally-active intergenic region between the *trnI–trnA* genes (in the *rrn* operon) located within the inverted repeat regions of the chloroplast genome [12,13,15–17], although several other sites have been explored (Figure 1B). With

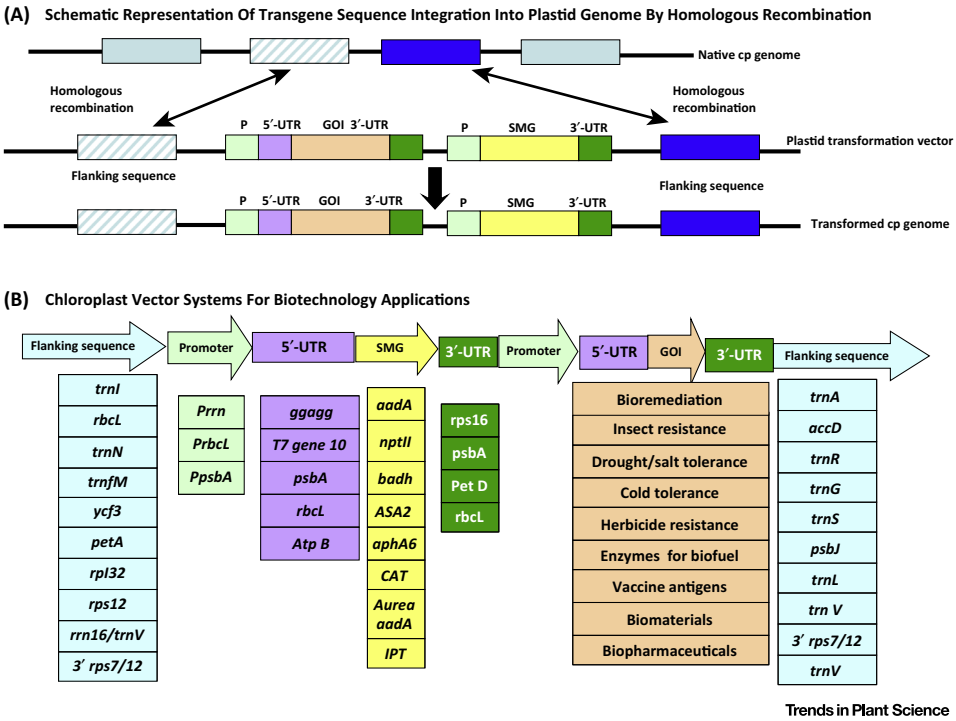


Figure 1. The Art of Chloroplast Genome Engineering. (A) Schematic representation of the chloroplast vector. The vector includes at least two chloroplast DNA fragments as flanking sequences to facilitate insertion by double homologous recombination, a selectable marker gene, and regulatory elements. (B) Examples of components commonly used in chloroplast vectors. Abbreviations: cp, chloroplast; GOI, gene of interest for various biotechnology applications; P, promoter; SMG, selective marker gene. 3′-UTR, 3′ untranslated region, used to enhance transcript stability; 5′-UTR, 5′ untranslated region, used to enhance ribosome binding.

Glossary

Blood–brain and blood–retina barriers: highly-selective permeability barriers that separate the circulating blood from the extracellular fluid in the central nervous system or retina, and which only allow the passage of small molecules (water, gas, glucose) and lipid-soluble molecules by passive diffusion.

Cholera toxin B (CTB): binds to receptors for GM1 ganglioside on the surface of target cells. Once bound, the entire toxin complex and fused proteins are endocytosed by that cell. CTB is therefore used as a fusion tag to facilitate protein drug delivery.

CO_2 concentrating mechanism (CCM): an effective adaptation that increases the CO_2 concentration around the primary photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

Cpn60 and Hsp93: heat-shock proteins of different molecular sizes (60 and 93 kDa) that are generally responsible for preventing thermal damage to proteins.

Homoplasmy: describes the presence of only one type of chloroplast genome in a genetically modified plant cell.

Immunogobulins IgA and IgG1: antibodies that develop after immunization and that offer protection against invading pathogens by binding to their surface proteins.

Inner envelope protein 37 kDa (IEP 37): this chloroplast protein is part of the protein import machinery in the TIC–TOC (translocon inner/outer membrane complexes) system.

Inner membrane (IM) of the chloroplast envelope: chloroplasts have a double membrane system: the inner membrane and the outer membrane.

Intercistronic expression element (IEE): optional element in plastids that produces monocistronic mRNAs from polycistronic mRNAs.

Interleukin 10 (IL-10): also known as human cytokine synthesis inhibitory factor (CSIF), IL-10 is an anti-inflammatory cytokine.

Light-harvesting chlorophyll protein (LHCP): the LHCPa/b protein is an integral membrane protein.

Oxygen-evolving protein (OE23): the 23 kDa subunit of the oxygen-evolving complex is located deeply

insertion of seven transgenes at this site, up to 13 genes could be driven by two endogenous 16S *rrn* and *psbA* promoters [18,19]. The flanking sequences include the chloroplast origin of replication (that provides more copies of templates for integration) and a copy-correction mechanism within the inverted repeat regions that enhances **homoplasmy** (see Glossary) [20]. In addition, introns present within these genes facilitate efficient processing of transgene transcripts. Transgene expression levels inserted at this site are among the highest reported [21–23].

It is important to use species-specific endogenous regulatory sequences in transgene cassettes to achieve high levels of expression [23]. The endogenous *psbA* promoter and 5′ and 3′ untranslated regions (UTRs) [24], and the heterologous bacteriophage T7 gene 10 [20,25], continue to be widely used for transgene expression, but more regulatory sequences are needed for multigene engineering. A chloroplast gene expression system driven by an inducible promoter would reduce the pleiotropic effects of toxic foreign proteins expressed in transplastomic plants. Such an inducible plastid gene expression system was first developed using a inducible nuclear promoter. The T7 RNA polymerase was targeted to chloroplasts to drive transgenes integrated into the chloroplast genome [25], and was used to express the *phb* operon a decade later [26]. More recently, the **riboswitch** concept has been introduced to regulate transgene expression in transplastomic plants [27]. Although this concept represents a simple approach to turn an introduced transgene on or off, the switching efficiency is low and the modulation of transgene expression after ligand addition or removal is poor; this strategy requires further optimization for enhanced transgene expression.

Two new strategies for the construction of chloroplast vectors have been developed recently. One method uses the Gateway system to simplify vector construction and improve vector design [28]. Another group used modular design of genetic elements to construct chloroplast vectors to build transcriptional units as well as target any homologous recombination site of choice [29]. In addition, an **intercistronic expression element** (IEE) was introduced into the spacer region between cistrons to enhance processing of polycistronic mRNA into monocistronic mRNA to enhance translation [7]. However, this concept contradicts recent in-depth ribosome profiling studies [30] that show similar translation efficiency in both spliced and unspliced native chloroplast polycistronic mRNAs, as demonstrated previously by high-level expression of several heterologous polycistrons via the chloroplast genome lacking an IEE [21,31], or multigenes engineered recently via the chloroplast genome [18,19]. New PCR methods using overlapping primers have been used to remove introns and permit the expression of eukaryotic genes without the need for cDNA libraries; this concept was successfully employed to transform the chloroplast genome with fungal genes containing >10 introns [32]. Although codon optimization is desirable to enhance the expression level of eukaryotic genes [33], the highest levels of expression have, ironically, been obtained with native human gene sequences [23], suggesting that codon usage in chloroplast is much more flexible than in several other recombinant protein expression systems.

One major limitation is the availability of selectable markers that impact only on chloroplast protein synthesis and not on any other cellular compartment. The *aadA* gene, first successfully used for *Chlamydomonas* chloroplast transformation [34], and later in tobacco chloroplasts [35] on spectinomycin selection, is the only marker that has worked reproducibly to regenerate transplastomic events in several different plant species. In recent years, several antibiotic-free selectable markers based on D-amino acid oxidase [36], isopentenyl transferase (IPT) [37], and the asanthranilate synthase ∝-subunit (ASA2) [38] have been developed (Figure 1B). Removal of the selectable markers can be achieved using direct repeats or Cre-*lox* recombination approaches [39]. Indeed, precise excision of a selectable marker gene (*aadA*) was accomplished recently from the most commonly used transgene integration site (*trnA*/*trnI*) by using the mycobacteriophage Bxb1 recombinase and *attP*/*attB* recognition sites [40].

inside chloroplasts within the thylakoid lumen.
Phosphoenolpyruvate translocator (PPT): part of protein import machinery located in the inner chloroplast envelope membrane.
Renin–angiotensin system (RAS): this system plays an important role in cardiovascular homeostasis and in the pathogenesis of inflammation and autoimmune dysfunction. Angiotensin II (Ang II, processed by angiotensin converting enzyme, ACE) functions as a proinflammatory effector via the angiotensin type 1 receptor. RAS imbalance results in development of pulmonary hypertension, retinal diseases, and muscular dystrophy.
Regulatory T cells (Tregs): T cells that express immunosuppressive cytokines.
Retrograde signaling: signaling between different subcellular organelles and the nucleus; protein expression in the chloroplast has been shown to regulate nuclear gene expression.
Riboswitch: a regulatory fragment of mRNA that binds to its effectors, resulting in changes in its own activity.
Transforming growth factor β (TGF-β): an immunosuppressive cytokine involved in induction of tolerance.
Tic110, Tic40, Toc159: protein translocons located at the inner (TIC) and outer (TOC) chloroplast envelope membranes.
γ-Tocopherol methyltransferase (γTMT): catalyzes the last step of ∝-tocopherol biosynthesis and converts γ-tocopherol to ∝-tocopherol.
Total soluble protein (TSP): the total content of soluble protein in leaf extracts.

In the past two decades chloroplast genetic engineering has focused primarily on achieving hyperexpression of foreign proteins. Although the chloroplast genome can produce abundant transcripts, it has not yet been exploited to produce and deliver double-stranded (ds) RNA. Application of RNAi technology via the plant nuclear genome has several limitations. Likewise, delivery of small RNA prepared in other systems for human therapeutics is highly challenging [41]. In agriculture, there is a great need to downregulate harmful genes to protect plants from pests. Similarly, downregulation of dysfunctional genes that cause cancer, autoimmune diseases, or immune disorders could help in their treatment. Owing to the high level of chloroplast transcription, dsRNA could be synthesized in large quantities and orally delivered via bio-encapsulation in plant cells to target harmful genes [42]. In the examples described below, β-actin, lepidopteran chitin synthase *(Chi)*, cytochrome P450 monooxygenase (*P450*), and *V-ATPase* dsRNA made in chloroplasts were used to silence these target genes in the insect gut [10,43].

Emerging New Concepts for Insect Control via the Chloroplast Genome

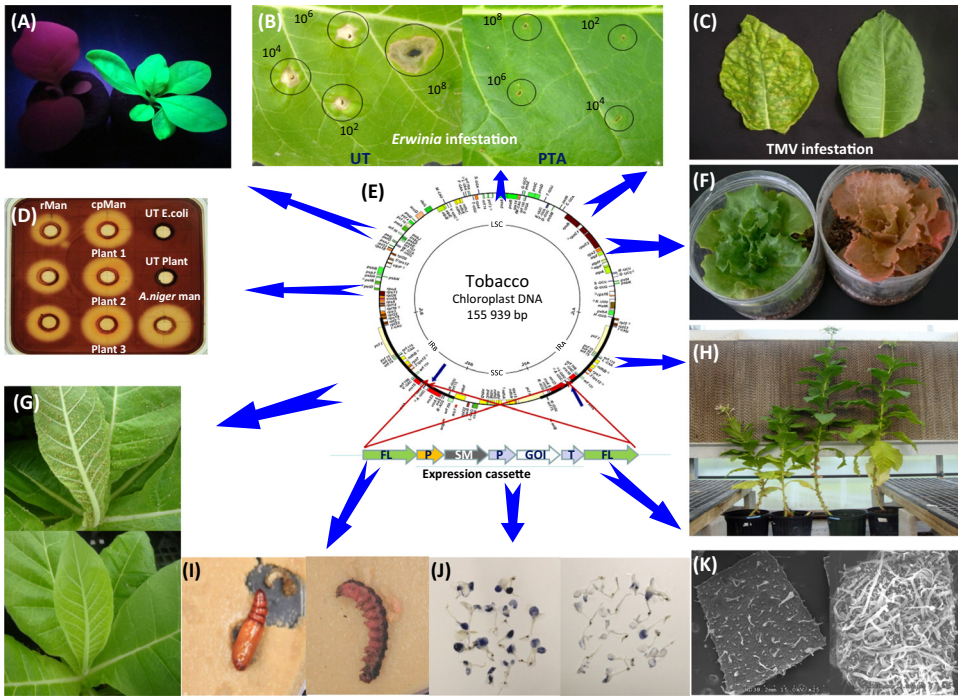
Although major advances have been made in hyperexpressing native biopesticide genes from *Bacillus thuringiensis* via the chloroplast genome to form crystals of the *B. thuringiensis* toxin Bt within chloroplasts [21], and even kill 40 000-fold resistant insects [44], plastid expression of *Bt* genes in important major crops [45] has not yet reached commercial development, largely because the market is already saturated with Bt crops that avoid the use of expensive chemical pesticides. However, a recent report of alarming Bt resistance has led to new US Environmental Protection Agency (EPA) requirements for planting Bt corn [46], and highlights the need for a high dose or multigene strategy. Thus, recent focus in this field is shifting to identify novel traits or methods to facilitate commercial development.

Recently, the RNA interference (RNAi) concept was used for the first time to engineer the chloroplast genome (Figure 2I) [43]. In this study the lepidopteran chitin synthase *(Chi)*, cytochrome P450 monooxygenase (*P450*), and *V-ATPase*, were used as RNAi targets. The abundance of cleaved dsRNA was greater than that of the highly expressed endogenous *psbA* transcript. In insects feeding upon leaves expressing *P450*, *Chi*, and *V-ATPase* siRNAs, transcript levels of the targeted genes were reduced to almost undetectable levels in the insect midgut, most likely after further processing of siRNA in the insect gut. The net weight of the larvae as well as growth and pupation rates were significantly reduced (Figure 2I) [43]. In a parallel study, Bock and colleagues introduced dsRNA via the chloroplast genome to target the insect *β-actin* gene and elicit resistance against potato beetle; this outstanding work demonstrated efficacy against this important pest in field studies [10]. Taken together, successful expression of dsRNAs via the chloroplast genome opens the door to the use of RNAi approaches to confer desired agronomic traits or to downregulate dysfunctional genes in various biomedical applications following oral delivery of dsRNA bio-encapsulated within plant cells.

Broad-Spectrum Agronomic Traits Conferred via the Chloroplast Genome

Expression of β-glucosidase in chloroplasts has been developed as a novel method to release active hormones (gibberellin, indolyl-3-acetic acid, zeatin) from inactive ester conjugates. Transplastomic lines showed increased leaf area, height, biomass and internode length. Most importantly, the density of globular trichomes containing sugar esters on the leaf surface was dramatically increased, conferring protection against whitefly and aphid infestations. These novel observations open new avenues to modify plants for enhanced biomass and to confer novel traits such as insect resistance (Figure 2H,K) [47].

In addition, transplastomic plants expressing the *Pinellia ternata* agglutinin (*pta*) showed broad-spectrum resistance to phloem-feeding pests as well as antiviral and antibacterial activity, providing a new option to engineer protection against different types of biotic stress using a single protein that is naturally present in medicinal plants (Figure 2B,C,G) [48]. Furthermore,



Trends in Plant Science

Figure 2. Engineering the Chloroplast Genome to Confer Biotic/Abiotic Stress-Tolerance or Expression of High-Value Products. (A) Antimicrobial peptide retrocyclin-101 fused with GFP expressed in chloroplasts displays strong green florescence in contrast to untransformed leaves showing chlorophyll red fluorescence [50]. (B,C) The transplastomic leaf expressing *Pinellia ternata* agglutinin shows high-level tolerance when challenged with bacterial (*Erwinia*) or viral (tobacco mosaic virus, TMV) pathogens [48]. (D) Gel diffusion assay shows the zone of clearance of chloroplast-derived endo-β-mannanase in crude plant extracts similar to purified recombinant commercial enzyme [52]. (E) Tobacco chloroplast genome and integration of the expression cassette. (F) Enhanced accumulation of astaxanthin and carotenoids in transplastomic lettuce [55]. (G) Transplastomic plants expressing the lectin gene show broad-spectrum resistance to lepidopteran and homopteran (sap-sucking) insects as well as antibacterial (*Erwinia*) and antiviral (TMV) activities [48]. (H,K) Chloroplast expression of β-glucosidase results in elevated phytohormone levels associated with significant increase in biomass and trichome density [47]. (I) Cotton bollworm larvae with normal pupation or dead larvae when fed with transplastomic tobacco leaves expressing dsRNAs [43]. (J) Overexpression of γ-tocopherol methyl transferase chloroplasts confers abiotic stress tolerance and nutritional enhancement [56].

'gene stacking' (combining two or more genes of interest) using protease inhibitors and chitinase has been reported in tobacco; transplastomic tobacco displayed broad-spectrum resistance against insects, pathogens, and abiotic stress [49]. A list of recent reports on enhancing agronomic traits via the chloroplast genome is provided in Table 1. Other novel approaches used to engineer protection against viral, bacterial, or fungal pathogens include the expression of synthetic antimicrobial peptide genes [50,51] (Figure 2A) or bacterial/fungal enzymes [52,53], as described below.

Metabolic Engineering via the Chloroplast Genome

In the first metabolic engineering study, bacterial chorismate pyruvate lyase was expressed from the chloroplast genome; plants accumulated *p*-hydroxybenzoic acid liquid crystal polymers up to 26.5% of dry weight with no pleiotropic effects [54]. Recently, the entire cytosolic mevalonate pathway encoding six enzymes combined with a selectable marker (seven genes in total) were inserted into the tobacco chloroplast genome. Despite lack of enhanced regulatory sequences (promoters/UTRs), transplastomic plants accumulated significantly higher levels of mevalonate, carotenoids, squalene, sterols, and triacylglycerols than control plants, successfully redirecting metabolic fluxes for isoprenoid biosynthesis [18].

Table 1. Recent Reports on Enhancement of Agronomic Traits Engineered via the Chloroplast Genome

Protein/enzyme	Source of Transgene	Plant Species	Expression Levels/Activities	Function/Phenotype	Refs
Retrocyclin-101 and protegrin-1	Synthetic	Tobacco	32–38% and 17–26% total soluble protein in leaves (TSP)	Enhanced resistance to *Erwinia* soft rot and TMV	[50]
β-Glucosidase Bgl1	*T. reesei*	Tobacco	Up to 44.41 units/g leaf	Whitefly and aphid resistance	[47]
Metallothionein-1	Mouse	Tobacco	183 000 transcripts/ng of RNA	Phytoremediation by Hg chelation	[93]
D-amino acid oxidase	*Schizosaccharomyces pombe*	Tobacco	Not reported (NR)	D-alanine-based herbicide resistance	[36]
Agglutinin	*Pinellia ternata*	Tobacco	7.1–9.2% TSP	Multiple resistance against aphid, whitefly, lepidopteran insects, and bacterial and viral pathogens	[48]
Thioredoxin f	Tobacco	Tobacco	NR[a]	Enhanced starch accumulation in leaves	[94]
Toc cyclase and γ-tocopherol methyltransferase	*Arabidopsis*	Tobacco and lettuce	3.05 nmol h^{-1} mg^{-1} protein	Enhanced vitamin E accumulation in tobacco and lettuce	[9]
Homogentisate phytyltransferase tocopherol cyclase, and γ-tocopherol methyltransferase	*Synechocystis* sp. PCC6803	Tomato	NR	Enhanced vitamin E accumulation in fruits. Increased light and cold stress tolerance	[7]
β,β-Carotenoid-3,3′-hydroxylase, β,β-carotenoid 4, 4′-ketolase (4,4′-oxygenase)	*Brevundimonas sp.* strain SD212	Lettuce	NR	Increased astaxanthin fatty acid esters accumulation in lettuce plants	[55]
Thioredoxins m	Tobacco	Tobacco	NR	Enhanced resistance to oxidative stress in tobacco plants	[95]
γ-Tocopherol methyltransferase	*Arabidopsis*	Tobacco	7.7% of the total leaf protein	Enhanced accumulation of α-tocopherol in seeds. Increased salt and heavy metal tolerance	[56]
Protease inhibitors and chitinase	*Paecilomyces javanicus*	Tobacco	NR	Broad-spectrum resistance against insects, pathogens, and abiotic stresses	[49]
Chloroperoxidase (CPO)	*Pseudomonas pyrrocinia*	Tobacco	15 μg CPO/ml extract	Enhanced resistance to fungal pathogens *in vitro* and *in planta*	[96]

[a]NR, not reported.

The natural pigment astaxanthin has attracted recent attention in view of its antioxidant activity and color – astaxanthin underlies the red coloration of salmon and other organisms. Expression of *CrtW* (β-carotene ketolase), *CrtZ* (β-carotene hydroxylase) and isopentenyl diphosphate isomerase (*Idi*) gene from marine bacteria via the lettuce chloroplast genome led to the accumulation of astaxanthin fatty acid esters and other key carotenoids (artificial ketocarotenoids corresponded to 95% of total carotenoids) (Figure 2F) [55]. Tocopherols (the main forms of vitamin E) are lipid-soluble antioxidants and play an important role in the plant antioxidant network by eliminating reactive oxygen species (ROS). Expression of **γ-tocopherol methyltransferase** (γ-TMT) and tocopherol cyclase (TC) genes in chloroplasts resulted in α-Toc as a major isoform and increased total tocopherol levels [9]. Similarly, expression of HPT (homogentisate phytyltransferase), TC, and γ-TMT confirmed HPT as the rate-limiting enzymatic step and increased total tocochromanol 10-fold [7]. More recently, γ-TMT gene expression resulted in massive proliferation of the inner chloroplast envelope membrane [56]. High-level accumulation of α-Toc in transplastomic plants not only increased the nutritional value of plant but also enhanced tolerance to abiotic stress by decreasing ROS (Figure 2J), lipid peroxidation, and ion leakage [56]. These findings offer new insight into the regulation of vitamins or complex metabolite biosynthesis and highlight the potential of chloroplast genetic engineering for the nutritional enhancement of edible plants (Figure 2F).

Enhancing Photosynthetic Efficiency via the Chloroplast Genome

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), the key enzyme in the Calvin cycle, has attracted attention for as a means to enhance carbon fixation efficiency, increase catalytic activity, and/or reduce photorespiration. Early studies involved relocation of the small subunit gene to the chloroplast genome to assemble fully functional Rubisco within chloroplasts [57]. More recent studies have focused on the expression of heterologous Rubisco subunits in chloroplasts. Most recently, a breakthrough was made by introducing the **CO_2-concentrating mechanism** (CCM) from cyanobacteria into transplastomic plants [58]. The native tobacco gene encoding the large subunit of Rubisco was knocked out by inserting the large and small subunit genes of the *Synechococcus elongates* Se7942 enzyme. Se7942 Rubisco and CcmM35 (a β-carboxysomal protein) hybrid assembly within chloroplasts resulted in higher rates of CO_2 fixation efficiency but slowed growth. This represents a key step towards improved photosynthesis by chloroplast genetic engineering. Whitney and colleagues have also enhanced photosynthesis and growth by coexpressing the Rubisco ancillary chaperone RAF1 together with Rubisco; this was reported to increase recombinant Rubisco biogenesis [59].

Chloroplast Bioreactors for Biofuel Enzymes

The need for sustainable and renewable energy sources is an important global challenge because of dwindling fossil fuel reserves and a growing population [60]. Production of cellulose-derived ethanol is currently limited by the lack of infrastructure, technology, and the high cost of enzymes. For most bioethanol process, 1 ton of biomass requires 15–25 kg cellulase [61], or 11 million filter paper units (FPU) of cellulase (around 19 kg), to yield 84 gallons of ethanol. More importantly, it is necessary to produce different types of enzymes and cocktails of enzymes to efficiently hydrolyze different types of biomass based on their polymer compositions. Therefore, the first and foremost requirement for ligno-cellulosic ethanol production is to develop an efficient enzyme production system for economical and rapid biomass depolymerization. High levels of expression and compartmentalization of toxic proteins within chloroplasts protect transgenic plants from pleiotropic effects, making the chloroplast an ideal bioreactor for industrial enzyme production.

Although single biofuel enzymes were expressed a decade ago [62], followed by other studies [15,16], total biomass hydrolysis was not feasible because of the number of enzymes required. A

major recent advance has been the development of chloroplast-derived enzyme cocktails for the production of fermentable sugars from different sources of ligno-cellulosic biomass. Most notably, nine different genes from bacteria or fungi (acetylxylan esterase, cutinase, endoglucanases, exoglucanase, pectate lyases, xylanase, lipase, etc.) were expressed in *Escherichia coli* or/and tobacco chloroplasts. The cost of chloroplast-derived endoglucanase was estimated to be 1000–3000-fold lower than for the same recombinant enzymes sold commercially [32]. This is the first report of using plant-derived enzyme cocktails for the production of fermentable sugars from ligno-cellulosic biomass.

However, overexpression of the enzymes (5–40% of total leaf protein) resulted in pigment-deficient mutant phenotypes, especially for enzymes such as swollenin and expansin that destabilize membranes [53]. Intertwined cotton fibers were irreversibly unwound when treated with chloroplast-derived swollenin. Likewise, recombinant cutinase effectively hydrolyzed digalactosyldiacylglycerol (DGDG) to monogalactosyldiacylglycerol (MGDG), showing α-galactosidase activity and demonstrating DGDG as a novel substrate [53]. Mannan is the major backbone of woody biomass, and β-mannanase can efficiently catalyze endohydrolysis of this constituent. Therefore, the addition of chloroplast-derived mannanase to other enzymes in the cocktail further enhanced biomass hydrolysis [52], and gel diffusion assay for endo-β-mannanase confirmed the functionality of the chloroplast-derived enzyme (Figure 2D). Another advance is the production of thermostable enzymes in chloroplasts to enable biomass hydrolysis [63]. A list of recent chloroplast-derived biofuel enzymes is summarized in Table 2. These studies are promising and have advanced the use of chloroplast-derived enzyme cocktails for biofuel production.

Chloroplast Bioreactors for Biopharmaceuticals

The first biopharmaceutical (a recombinant protein) expressed from recombinant plants is now FDA approved and marketed by Pfizer [64]. Recombinant glucocerebrosidase made in carrot cells is now used as a replacement therapy to treat Gaucher's disease, a rare lysosomal storage disorder. More recently, plant-based production of the Ebola vaccine (three humanized monoclonal antibodies) has been used successfully to treat infected individuals in the West African outbreak [64,65]. These protein drugs lead the way for producing biopharmaceuticals in plants. Biopharmaceuticals produced in current fermentation systems are very expensive and are not affordable for the large majority of the global population. In the USA, the average annual cost of protein drugs is 25-fold greater than for small-molecule drugs. This is because their production requires prohibitively expensive fermenters, purification, cold storage, and sterile delivery methods (via injection). However, oral delivery of protein drugs in genetically modified plant cells is now emerging as a new platform for inducing tolerance against autoimmune disorders, to eliminate the toxicity of injected protein drugs, or to deliver functional blood proteins [66–69]. Plant cells expressing high levels of therapeutic proteins can be lyophilized and stored at room temperature for several years [42]. These approaches should improve patient compliance in addition to lowering the cost of healthcare.

These studies point out the importance of oral delivery of protein drugs, which has been elusive for decades because of their degradation in the digestive system and inability to cross the gut epithelium. The first concern has been addressed by expression of protein drugs via the chloroplast genome in edible plant cells, taking advantage of the several thousand genome copies present in each plant cell. Although early efforts to express therapeutic proteins in lettuce chloroplasts were unsuccessful [70], extensive optimization was undertaken to develop a reproducible expression system utilizing species-specific chloroplast vectors, endogenous regulatory sequences, and optimal organogenesis/hormone concentrations to directly generate transplastomic lines without callus induction [23]. Today, lettuce serves as the only reproducible transplastomic system for oral delivery of vaccines and biopharmaceuticals (Table 3).

Table 2. Chloroplast Bioreactors for Proteins/Enzymes for Biofuel Production

Protein/Enzyme	Source of Transgene	Enzyme Activity	Refs
β-Glucosidase Swollenin Xylanase Acetyl xylan esterase Endoglucanase Endoglucanase Exoglucanase Lipase Pectate lyase A Pectate lyase A Pectate lyase A Cutinase	*Trichoderma reesei* *T. reesei* *T. reesei* *T. reesei* *T. reesei* *C. thermocellum* *C. thermocellum* *M. tuberculosis* *F. solani* *F. solani* *F. solani* *F. solani*	Chloroplast-derived enzymes showed wider pH optima and higher temperature stability than enzymes expressed in *E. coli*. Chloroplast-derived crude-extract enzyme cocktails yielded more (up to 3625%) glucose from citrus peel, filter paper, or pine wood than commercial cocktails	[32]
β-Glucosidase BglC	*T. fusca*	Chloroplast-produced BglC was active against both cellobiose and lignocellulose	[88]
β-Glucosidase Bgl1	*T. reesei*	Chloroplast-produced Bgl1 can digest pNPG substrate and release *p*-nitrophenol	[47]
Xylanase Xyl10B	*T. maritima*	Catalytic activity of chloroplast-derived Xyl10B in poplar, sweetgum and birchwood xylan and stable in dry leaves	[63]
β-Glucosidase Bgl1C Endoglucanase Cel9A Exoglucanase Cel6B Xyloglucanase Xeg74	*T. fusca* *T. fusca* *T. fusca* *T. fusca*	All four enzymes were highly active and hydrolyzed their synthetic test substrates in a dose-dependent manner. The enzyme cocktail also triggered efficient sugar release from straw	[97]
β-Mannanase	*T. reesei*	Chloroplast-derived mannanase showed 6–7-fold higher enzyme activity than *E. coli* extracts. The enzyme cocktail with chloroplast -derived mannanase yielded 20% more glucose equivalents from pinewood than the cocktail without mannanase	[52]
Cutinase or swollenin	*Fusarium solani* *T. reesei*	Cotton fiber treated with chloroplast-derived swollenin showed enlarged segments, and the intertwined inner fibers were irreversibly unwound due to the expansin activity of swollenin. Chloroplast-derived cutinase showed esterase and lipase activity	[53]
β-1, 4-Endoglucanase (EGPh)	*Pyrococcus* horikoshii	Chloroplast-derived EGPh was recovered from dry leaves and digested carboxymethyl cellulose (CMC) substrate	[98]
Xylanase	*Bacillus* sp.	Catalytic activity of chloroplast-produced xylanase was detected with birch wood xylan as substrate	[99]

Upon oral delivery, the plant cell wall protects expressed protein drugs from acids and enzymes in the stomach via bio-encapsulation. However, when intact plant cells containing protein drugs reach the gut, commensal microbes are able to digest the plant cell wall and release the protein drugs. In addition, tags such as CTB (**cholera toxin B**) bind to the GM1 (monosialotetrahexosylganglioside) receptor expressed on the gut epithelium; fusion of CTB to protein drugs leads to efficient crossing of the intestinal epithelium and delivery to the circulatory or immune system. In addition, the GM1 receptor is also expressed in the retina and nervous system, and can aid tagged protein drugs to cross the **blood–brain** (BBB) and **blood–retina barriers** (BRB), thereby facilitating delivery to the brain and retina [66,74]. These steps are explained in detail in Figure 3. More than 40 biopharmaceuticals and vaccine antigens have been expressed via the chloroplast genome (Table 3, Figure 3).

Table 3. Chloroplast Bioreactors for Functional Biopharmaceuticals and Vaccine Antigens

Biopharmaceutical/Vaccine Antigen	Expression System	Expression Level	Functional Evaluation	Refs
CTB–AMA1 (malarial vaccine antigen apical membrane antigen-1)	Lettuce Tobacco	7.3% TSP 13.2% TSP	Long-term dual immunity against two major infectious diseases – cholera and malaria	[79]
CTB–MSP1 (malarial vaccine antigen merozoite surface protein-1)	Lettuce Tobacco	6.1% TSP 10.1 TSP	Long-term dual immunity against two major infectious diseases – cholera and malaria	[79]
EDA (extra domain A-fibronectin)	Tobacco	2.0% TSP	Retains the proinflammatory properties of the EDA produced in *E. coli*	[100]
Parvovirus immunogenic peptide 2L21 fused to a tetramerization domain	Tobacco	6% TSP	Immunogenic response in mice	[101]
Immunogenic fusion protein F1-V from *Y. pestis*	Tobacco	14.8% TSP	Oral delivery of F1-V protected 88% of mice against aerosolized *Yersinia pestis*; F1-V injections protected only 33% and all control challenged mice died. Oral boosters conferred protective immunity against plague	[80]
Coagulation factor IX	Tobacco	3.8% TSP	Prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice	[76]
BACE (human β-site APP cleaving enzyme)	Tobacco	2.0% TSP	Immunogenic response against the BACE antigen in mice	[102]
Human papillomavirus L1 protein	Tobacco	21.5% TSP	Confirmed the formation of capsomeres	[103]
Proinsulin	Tobacco	47% TSP	Oral delivery of proinsulin in plant cells or injectable delivery into mice showed reduced blood glucose levels	[71]
PA(dIV) (domain IV of *Bacillus anthracis* protective antigen)	Tobacco	5.3% TSP	Demonstrates protective immunity in mice against anthrax	[104]
Human thioredoxin 1 protein	Lettuce	1% TSP	Protected mouse insulinoma line 6 cells from hydrogen peroxide	[105]
Thioredoxins–human serum albumin fusions	Tobacco	26% TSP	The *in vitro* chaperone activity of Trx m and f was demonstrated	[92]
HPV16 L1 antigen fused with LTB	Tobacco	2% TSP	Proper folding and display of conformational epitopes	[106]
Exendin 4 (EX4) fused to CTB	Tobacco	14.3% TSP	CTB–EX4 showed increased insulin secretion similar to the commercial EX4 in β-TC6	[67]
CTB–ESAT-6 (6 kDa early secretory antigenic target)	Tobacco Lettuce	Up to 7.5%; 0.75%	Hemolysis assay and GM1-binding assay confirmed functionality and structure of the ESAT-6 antigen	[81]
CTB–Mtb72F (a fusion polyprotein from two tuberculosis antigens, Mtb32 and 39)	Tobacco	Up to 1.2%	Not reported	[81]
CTB fused to MBP (myelin basic protein)	Tobacco	2% TSP	Amyloid loads were reduced *in vivo* in brain regions of 3×TgAD mice fed with bio-encapsulated CTB–MBP. Aβ(1–42) accumulation was reduced in retinae and loss of retinal ganglion cells was prevented in 3×TgAD mice treated with CTB–MBP	[66]
	Tobacco	80 or 370 μg/g in fresh leaves	Feeding of the HC/C2 mixture substantially suppressed T helper cell	[78]

Table 3. (continued)

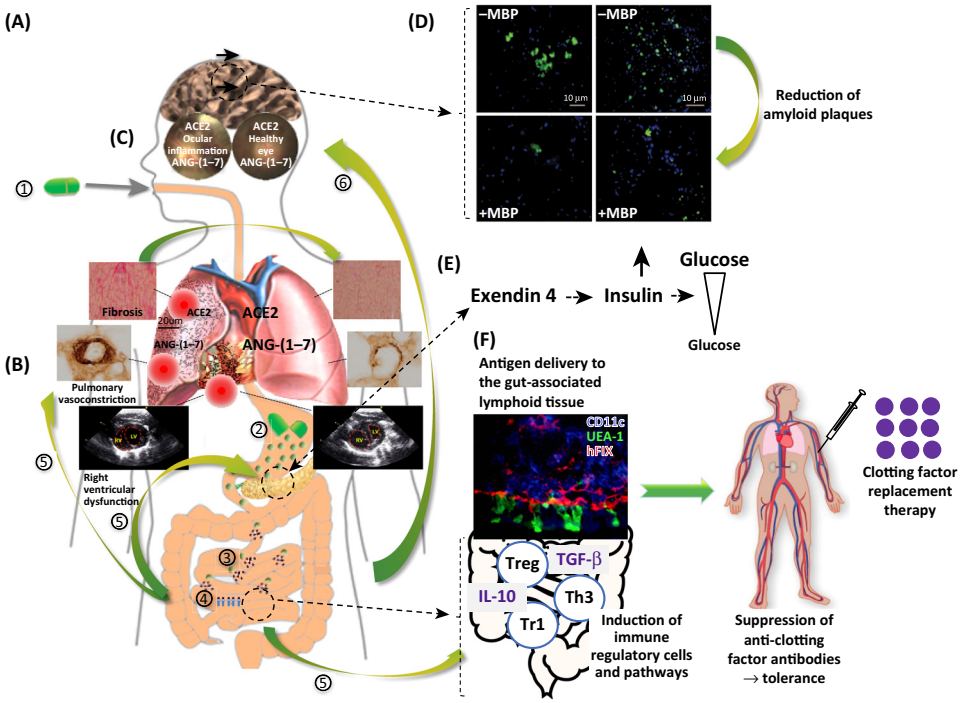


Figure 3. Oral Drug Delivery for Treatment of Metabolic Disorders or Induction of Tolerance. (A) Therapeutic proteins expressed in chloroplasts and bio-encapsulated in plant cells when orally delivered (step 1) go through the following process. (2) Protein drugs are protected from acids/enzymes in the digestive system because human enzymes do not break down glycosidic bonds of plant cell wall components. (3) Microbes colonizing the gut break down the plant cell wall, releasing therapeutic proteins into the gut. (4) The transmucosal carriers cholera toxin B (CTB) fused to protein drugs facilitates their delivery to the blood by binding to gut epithelial receptors for GM1. (5) Protein drugs in the blood are delivered to different organs including heart, lung, pancreas, and directly to immune modulatory cells. (6) Proteins fused with CTB also cross the blood–brain or blood–retina barriers via GM1 receptors present in these barriers. (B) Metabolic disorders caused by unbalanced renin–angiotensin system are prevented or delayed by oral delivery of bio-encapsulated angiotensin converting enzyme 2 (ACE2) and angiotensin-(1–7) [Ang-(1–7)]. Delivery of ACE2 and Ang-(1–7) to circulatory system reversed or prevented pulmonary hypertension by shifting the RAS axis to a protective state, resulting in decrease of fibrosis, improvement of cardiopulmonary structure and function, and restoration of right heart function. Oral protein drug delivery across the blood–brain and blood–retina barriers: (C) Ocular inflammation caused by decreased activity of the protective axis of RAS was improved by oral delivery of bio-encapsulated ACE2 and Ang-(1–7) across the blood–retina barrier that entered the retina and reduced endotoxin-induced uveitis and experimental autoimmune uveoretinitis. (D) Likewise, oral delivery of myelin basic protein fused with CTB (CTB–MBP) entered the brain by crossing the blood–brain barrier and reduced Aβ plaques in advanced Alzheimer's brain. (E) Oral delivery of exendin expressed in chloroplasts increased insulin secretion and regulated blood sugar levels. (F) Oral tolerance induction: blood coagulation factor expressed in chloroplasts delivered into gut-associated lymphoid tissue (GALT) of hemophilia A mice induces immune regulatory cells and pathways. Upregulation of Treg markers (CD25, FoxP3, CTLA-4) and suppressive cytokines (IL-10 and TGF-β) is observed in hemophilia A mice fed with plant FVIII. After induction of oral tolerance, injection of recombinant FVIII into hemophilia A mice suppressed anti-clotting factor antibodies.

Type 2 diabetes is more prevalent than type 1 and affects significant proportion of the global population; a cost-effective treatment will be needed to deal with this global pandemic. Oral delivery of human proinsulin, expressed in chloroplasts and bio-encapsulated in plant cells (or purified from chloroplasts and delivered by injection), into mice decreased blood glucose levels with a similar efficiency to commercial insulin treatment [71].

Glucagon-like peptide (GLP-1) increases insulin secretion, but this peptide has a very short half-life (only 2 min in the circulatory system). The exenatide (an analog of GLP-1) has a much longer

half-life (3.3–4 h) and strong insulinotropic effects, but requires cold storage and daily abdominal injections with short shelf-life [67]. Oral delivery of chloroplast-derived CTB–EX4 (CTB fused to exendin 4) increased insulin secretion similarly to commercial EX4 (Figure 3A,E), which could eliminate injections, increase patient compliance/convenience, and significantly lower the cost of treatment [67].

Antimicrobial peptides (AMP) have an advantage over current antibiotics because they are effective against drug-resistant microbes. However, the high cost of producing AMPs is a major barrier for their clinical development and commercialization. Therefore, two important AMPs: retrocyclin 101 (RC-101) and protegrin 1 (PG1), were expressed in chloroplasts [50] for clinical development. Despite the requirement for complex post-translational modifications including cyclization, both AMPs were active against bacterial and viral pathogens. Transplastomic plants showed normal growth. Similarly, a phage lytic protein was also expressed in transplastomic tobacco plants and accumulated to high levels (>70% of **total soluble protein**, TSP); however, transplastomic plants showed retarded growth [72]. In a follow-up study a toxin shuttle strategy was used to address this concern [73].

Delivering neuro-therapeutics to target brain-associated diseases is a major challenge. Alzheimer's disease (AD) is the most common neurodegenerative genetic disorder and the sixth leading cause of death in the USA, affecting ~5.4 million Americans and 36 million patients globally. Oral delivery of CTB fused with myelin basic protein (MBP) to healthy and AD model mice increased MBP levels in different regions of the brain, effectively crossing the BBB. When sections of human and mouse AD were incubated with CTB–MBP *ex vivo*, the intensity of amyloid plaque was reduced by up to 60%. Moreover, bio-encapsulated CTB–MBP treatment *in vivo* decreased amyloid loads by 70% in the cortex and hippocampus of AD mice. CTB–MBP oral delivery also reduced accumulation of plaque in retinae. Thus, low-cost oral delivery of therapeutic proteins across the blood–brain and blood–retina barriers has been demonstrated (Figure 3A–D) [66].

Retinal inflammation is the main cause of visual impairment and is responsible for several retinal diseases. In the USA, 5–15% of all blindness is caused by uveitis, an intraocular inflammatory disorder. The protective axis of the RAS (**renin–angiotensin system**) was activated by oral delivery of chloroplast-derived angiotensin converting enzyme 2 (ACE2) and angiotensin 1–7 [Ang-(1–7)] fused to CTB; this was found to confer protection against ocular inflammation (Figure 3A). With this treatment, retinal vasculitis, cellular infiltration, and damage were dramatically decreased in experimental autoimmune uveoretinitis (EAU) [74] (Figure 3 A,C), further confirming that CTB facilitates delivery of protein drugs across the blood–retina barrier.

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by increased blood pressure in the pulmonary arteries. Oral delivery of plant cells expressing ACE2 or Ang-(1–7) significantly improved cardiopulmonary structure and function in rats with monocrotaline (MCT)-induced PAH in both prevention and reversal protocols. Not only was the elevated right ventricular systolic blood pressure decreased but pulmonary blood flow was also improved (Figure 3 A,B) [69].

Chloroplast Bioreactor for Induction of Oral Tolerance

Long-term delivery of several protein drugs by injection can lead to adverse effects. One such complication is the development of antibodies to injected proteins, thereby neutralizing the effects of injected drug or, in some cases, leading to toxic antibodies (such as **immunoglobulin IgE**) that cause allergies, anaphylaxis, or even death. Treatment of the genetic disease hemophilia A or B is severely hampered by antibody ('inhibitor') formation against the infused therapeutic clotting factors [75].

Oral tolerance induced by coagulation factor antigens bio-encapsulated in plant cells is emerging as an alternative cost-effective and promising strategy to eliminate this problem while avoiding the side effects of immune-suppressive drugs. In a murine model of hemophilia B that mimics the human inhibitor and anaphylactic responses to coagulation factor IX (FIX) replacement therapy, repeated oral delivery of plant cells expressing human FIX fused to CTB effectively prevented pathogenic antibody formation against FIX, and this treatment eliminated fatal anaphylactic reactions that occurred after four to six exposures to intravenous FIX [76,77]. More recently, this approach was tested for hemophilia A, the most prevalent form of the disease with a high incidence of inhibitor formation. Oral delivery of a mixture of plant cells expressing either the entire heavy chain or the C2 domain of human coagulation factor VIII (FVIII), suppressed inhibitor formation against FVIII in two different strains of hemophilia A mice. This study provided the first evidence that the plant-based oral tolerance protocol could reverse pre-existing responses and generated data on the underlying tolerance mechanisms [78] (Figure 3A,F). Delivery of antigen to dendritic cells (in the lamina propria and Peyer's patches) throughout the small intestine results in a complex immune regulatory mechanism. Adoptive transfer studies showed that, in addition to $CD4^+CD25^+FoxP3^+$ **regulatory T cells** (Tregs), $CD4^+CD25^-LAP^+$ Tregs are induced that potently suppress antibody formation. These studies for the first time demonstrate the ability of LAP^+ Treg cells to suppress inhibitor formation. These cells are also the primary T cell source of the immune-suppressive cytokines **transforming growth factor β** (TGF-β) and **interleukin** (IL)-10 in response to coagulation factor antigen during oral tolerance. Because similar results were obtained for both FVIII and FIX antigen delivery, the same tolerance mechanism,may apply generally to the plant cell-based protocol [77,78].

Chloroplast Bioreactors for Infectious Disease Vaccines

Although the field of plant-based vaccines started two decades ago, with the promise of developing low-cost vaccines to prevent infectious disease outbreaks and epidemics around the globe, this goal has not yet been realized. There are several major technical hurdles to be overcome in achieving this goal, including inadequate levels of expression in edible plant systems and the failure of oral priming to induce adequate immunity against pathogens. Currently, no method is available to induce oral priming, and the only reproducible priming strategy is to deliver antigens by injection in association with adjuvants. However, the major advantage of the oral vaccination system is that it can stimulate both mucosal (**immunoglobulin IgA**) and systemic (**IgG1**) immunity; this is presently achieved by priming using vaccine antigens delivered by injection, followed by oral boosting with antigens bio-encapsulated in plant cells. In addition, oral antigen delivery requires fusion with transmucosal carriers to cross the gut epithelium for delivery to the immune system [79]. Very few vaccine candidates listed in Table 3 meet all these requirements, and therefore their efficacy has not been tested in suitable animal models or they failed such tests. By contrast, candidates that meet these criteria have successfully demonstrated efficacy as oral vaccines to boost the immune system and confer greater/prolonged protection against pathogen challenge. However, all these studies require priming by injection, and therefore are not entirely free of cold storage, and sterility requirements. Furthermore, only a few vaccine antigens have been expressed in edible crops (lettuce) [79–81], and those expressed in tobacco would face challenge by the FDA approval process because of concerns of nicotine in orally delivered drugs.

Chloroplast Genome Engineering Enables the Understanding of Complex Cellular Processes

Studies on the native chloroplast genome and endogenous regulatory sequences contribute greatly to our understanding of the molecular biology, physiology, and biochemistry of chloroplasts. Transplastomic lines contribute to resolving complex processes that are difficult to study in native systems or when such results are inconclusive. For example, it was challenging to determine the precise site of cleavage of transit peptides after import of precursor proteins into

chloroplasts, but this could be clearly resolved by expressing precursor proteins via the chloroplast genome, demonstrating that this step takes place in the stroma and not in the chloroplast envelope [2]. Most importantly, the role of nucleus-encoded cytosolic proteins that bind to regulatory sequences and their species specificity could be clearly studied using transgene expression [23]. For example, the regulatory sequence of lettuce *psbA* decreased transgene expression >90% in tobacco chloroplasts, and vice versa, underscoring the importance of the species-specificity of chloroplast regulatory sequences [23]. Such studies are not possible using native genes. This may explain the failure of several laboratories to transform unrelated crop species using tobacco chloroplast vectors. Likewise, details of the homologous recombination process and the deletion of mismatched nucleotides were evident using heterologous flanking sequences [23]. Translation of polycistronic mRNAs without the need for monocistronic processing has been studied using ribosome profiling [30]. However, the use of heterologous polycistrons engineered via the chloroplast genome has offered even more direct evidence for this process [21,31], and the insertion of replication origins into chloroplast vectors has offered further insight into minimal sequences required [24].

Plastid and nuclear genomes require frequent and accurate signaling to coordinate the assembly of multisubunit complexes or enzymes involved in biosynthetic pathways. The nucleus-encoded plastid protein subunits are regulated by anterograde signaling pathways, which have been studied in depth. Interestingly, plastid-derived signals can also coordinate the expression of nuclear genes encoding plastid-localized proteins via retrograde signaling [82,83]. Several recent publications reveal that both transcripts and proteins can be exported from plastids. For example, expression of Tic40 (a translocation protein of the import complex localized in the inner plastid envelope) via the chloroplast genome resulted in massive proliferation of the **inner membrane** (IM) (up to 19 layers in electron micrographs of transformed chloroplasts) without any impact on plant growth or reproduction. Consistent with IM proliferation, the expression of several other **inner envelope proteins** (**phosphoenolpyruvate translocator** PPT, **IEP 37**, **Tic110**) was upregulated, but none of the outer membrane (Toc159), stromal (**Hsp93**, **Cpn60**) or thylakoid (**light-harvesting chloroplast protein** LHCP, **oxygen-evolving protein** OE23) proteins were increased, suggesting specific retrograde signal(s) [84]. This phenomenon is highly reproducible and takes place in the absence of any environmental stress. Expression of γ-TMT (inserted into the IM) via the chloroplast genome again resulted in massive proliferation of the inner envelope membrane (up to eight layers, Figure 4A,B) [56]. When lectin or AMP genes were expressed via the chloroplast genome, they conferred broad protection against bacterial or viral pathogens [48,50]. Release of AMPs from chloroplasts could possibly be explained by lysis of plastids; indeed, retention of AMPs within intact plastids seems unlikely because invading pathogens are in the cytosol [50]. NRIP1 (a chloroplast-localized receptor interacting protein) was found to interact with a cytoplasmic P50 helicase enzyme following infection by tobacco mosaic virus (TMV) [85], and several nucleotide-binding receptors are localized within the chloroplasts. Caplan and his colleagues showed that most secreted proteins of *Pseudomonas syringae* contain chloroplast targeting signal sequences, and these proteins require retrograde signaling to the nucleus to elicit defense responses [86].

To date, the mechanistic details of the retrograde signaling pathway have remained elusive. There are several proposed retrograde signaling pathways [82,87], and a novel operational retrograde signaling pathway was recently described by Xiao *et al.* [83]. Methylerythritol cyclodiphosphate (MEcPP) is the precursor for the isoprenoids generated by the plastid methylerythritol phosphate (MEP) pathway, and MEcPP accumulation in plastids leads to activation of nuclear stress-responsive genes. The redox state of photosynthetic electron transport components and ROS levels in plastids may provide another important retrograde signal. Unfortunately, none of these proposed candidates to date have been unequivocally tested. To investigate the potential role of ROS in the retrograde signaling pathway and transmembrane transport,

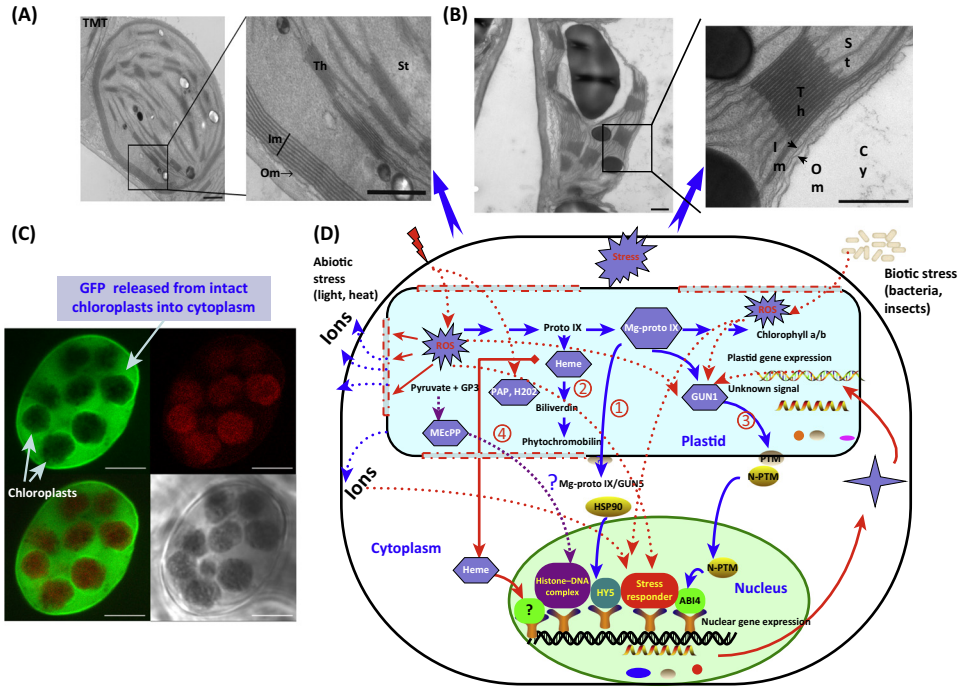


Figure 4. Chloroplast Genome Engineering Facilitates Study of Retrograde Signaling. (A) Expression of γ-tocopherol methyl transferase via the chloroplast genome results in massive proliferation of the inner envelope membrane (up to eight layers) [56], very similar to 19 layers observed when the inner membrane (IM) protein Tic40 was overexpressed [84]. While several IM proteins encoded by the nuclear genome are upregulated, none of the outer membrane, stromal, or thylakoid proteins are affected, suggesting specificity of retrograde signaling. (B) Note a single inner envelope membrane in control chloroplasts [56]. (C) GFP fused with an antimicrobial peptide (AMP) is released from intact chloroplasts when the leaf discs were infected with *Erwinia carotovora* and plant cells were imaged by confocal microscopy ([51] for time-lapse images of GFP–AMP). Transient release of GFP–AMP triggered by *Erwin*ia infection stops soon after conferring protection against the invading pathogen, further confirming protein export from chloroplasts. Light and paraquat stress modifies the structure of chloroplast envelope, leading to increased ion leakage and facilitates AMP release [51]. These results suggest novel retrograde signaling mechanisms triggered by foreign proteins expressed in chloroplasts and offer new opportunities to study pathways outside chloroplasts. (D) Proposed signaling mechanisms between plastid and nuclear genome are shown. (1) Mg-protoporphyrin IX (Mg-proto IX) pathway: Mg-proto IX is an intermediate of tetrapyrrole pathway, which is suggested to be exported from chloroplasts to bind to HSP90 in the cytoplasm, resulting in a HY5-dependent activated repression and/or inhibited activation of nuclear expression [87]; (2) Heme, specifically produced by the plastid ferrochelatase (FC1) has been suggested to coordinate photosynthesis-associated nuclear gene expression with chloroplast development [107]; (3) tetrapyrrole or other signals may work on genome uncoupled 1, which could either generate or transmit a second signal, thereby activating N-PTM protein. The processed PTM may modulate nuclear gene expression by inducing the ABA-insensitive 4 transcription factor [87]; (4) MEcPP (methylerythritol cyclodiphosphate), a precursor of isoprenoids produced by the plastid methylerythritol phosphate (MEP) pathway, accumulates during stress. MEcPP destabilizes histone-like protein–DNA complexes in bacteria, suggesting a possible model for gene regulation [82]. The redox state of photosynthetic electron transport components of the plastids and the levels of reactive oxygen species (ROS) in plastids may be another candidate for retrograde signaling. ROS accumulate promptly when plants cells are exposed to stress. ROS alter the membrane structure and increase membrane penetrability, which is helpful for the transmission of signal molecules from plastids. More importantly, ROS may be directly involved in other retrograde signaling pathways such as the MEcPP pathway as well as the plastid gene expression pathway [51,82].

transplastomic plants expressing a GFP–AMP fusion polypeptide were developed. This study revealed that chloroplast GFP is gradually released from intact chloroplasts into the cytoplasm after abiotic and biotic stress [51] (Figure 4C). Light and paraquat stress modified the structure of chloroplast envelope, resulting in increased ion leakage and facilitated protein release. Release of the AMP (GFP–RC101) triggered by *Erwinia* infection conferred protection against the

pathogen, further confirming protein export from the chloroplast [51]. These results suggest that novel retrograde signaling mechanisms may be triggered by chloroplast proteins and offer new opportunities to study pathways outside chloroplasts (Figure 4D). Thus chloroplast genetic engineering offers an ideal new tool to study interactions with other cellular compartments or within chloroplasts.

Concluding Remarks and Future Perspectives

Although hundreds of foreign proteins have been expressed in chloroplasts, and have often achieved much higher levels of expression than nuclear expression systems, in a few cases the desired levels of expression were not achieved. N-terminal degradation of proteins in heterologous systems is a well-known phenomenon. Indeed, the oldest and best-known recombinant human blood protein, insulin, has never been expressed in any expression system without N-terminal fusion proteins. Many human therapeutic proteins have therefore been successfully expressed in chloroplasts by fusion with GFP [50] to confer stability, or with CTB to facilitate stability and oral delivery [66–69]. Several upstream and downstream transcriptional and translational regulatory sequences have been used to enhance transgene expression via the chloroplast genome [15–17,88]. In addition, reduced expression could be due to misfolding of proteins. Indeed, this was clearly evident with human FIX fused with CTB with or without a furin cleavage site; when the furin cleavage site was eliminated, expression levels decreased 50-fold and homoplasmy could not be achieved [76]. For the expression of toxic proteins, inducible expression system would be ideal to synthesize foreign proteins only when needed, conserving cellular resources for normal growth and development. However, further research will be necessary to develop highly-efficient inducible systems in chloroplasts. Transformation of the lettuce chloroplast genome is the only reproducible system currently used for oral delivery of therapeutic proteins. Further studies will be needed to develop chloroplast transformation in other leafy edible systems that could be orally delivered with minimal processing. Most importantly, further studies are needed to understand post-translational modifications of proteins within chloroplasts. Recent studies have shown that human blood proteins with disulfide bonds (such as insulin, interferon, etc.) are properly folded and are fully functional in the chloroplast [71,89]. Chloroplasts are also capable of assembling multimeric structures (such as CTB) with disulfide bonds that bind to GM1 receptors [66–69,74,76,77]. Likewise, the assembly of virus-like particles has been observed in chloroplasts [90,91]. Protein disulfide isomerase/thioredoxin expression has been shown to enhance the folding and assembly of human serum albumin within chloroplasts [92]. Cyclization with disulfide bonds is required for the antimicrobial activity of retrocyclin, and chloroplasts synthesize and fold such cyclic proteins [50]. However, several complex post-translational modifications take place within chloroplasts. Human blood proteins correctly expressed in chloroplasts should facilitate our understanding of the hitherto unknown post-translational modifications that take place within chloroplasts.

There is enormous potential for synergistic utilization of chloroplast genome engineering with synthetic biology, opening ways to introduce entire genomes. While current approaches facilitate engineering pathways, introducing synthetic genomes would be revolutionary. Therefore, further research could advance chloroplast engineering towards clinical products, improved agronomic traits, metabolic engineering to produce novel fuels, enhanced nutrition, and also advance our understanding of basic cellular signaling and metabolic processes.

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Outstanding Questions

What are the limitations in transforming the chloroplast genome of cereals? While regeneration via somatic embryogenesis is feasible in dicots, homoplasmic transplastomic plants have not yet been created after two decades of research. Because proplastids or non-green plastids in carrot or soybean have been transformed successfully, it is unlikely that gene delivery or regeneration processes will provide a hurdle, but there is need to identify suitable selectable markers.

What are the limitations in transforming the chloroplast genome of new crop species? There is a great need to transform edible leafy crops, especially for oral drug delivery or enhanced nutrition. Edible non-green parts of plants (e.g., tomato fruits) have very low levels of foreign protein accumulation compared to transplastomic leaves. With the exception of lettuce chloroplasts, no other edible system has yielded reproducible results so far. Lessons learned from optimization of lettuce plastid transformation, especially species-specific chloroplast vectors and endogenous regulatory sequences, should offer some guidance.

Is it possible to achieve inducible expression? Constitutive expression of particular proteins can be problematic. Although the T7 RNA polymerase system was introduced in 1994, it is still challenging to regulate transgene expression and synthesize products on demand.

Can the chloroplast serve as bioreactor for RNA synthesis and delivery? While chloroplasts are ideal for protein expression and delivery, could this system be used for RNA silencing, a major need in agriculture and medicine? Indeed, chloroplasts are capable of producing highly-abundant transcripts, but heterologous RNA processing is poorly understood.

Can a synthetic plastome be engineered? Foreign operons or new pathways have been engineered via the chloroplast genome with great success, even including the formation of protein crystals. However, several challenges need to be overcome to use chloroplasts for synthetic biology applications, including the introduction of large DNA fragments.

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