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Chapter

Chloroplasts: The Future of Large-Scale Protein Production

Brenda Julian Chávez, Stephanie Solano Ornelas,
Quintín Rascón Cruz, Carmen Daniela González Barriga,
Sigifredo Arévalo Gallegos, Blanca Flor Iglesias Figueroa,
Luis Ignacio Siañez Estrada, Tania Siqueiros Cendón,
Sugey Ramona Sinagawa García and
Edward Alexander Espinoza Sánchez

Abstract

Chloroplast engineering has matured considerably in recent years. It is emerging as a promising tool to address the challenges related to food security, drug production, and sustainable energy posed by an ever-growing world population. Chloroplasts have proven their potential by efficiently expressing transgenes, encapsulating recombinant proteins, and protecting them from cellular machinery, making it possible to obtain highly functional proteins. This quality has also been exploited by interfering RNA technology. In addition to the practical attributes offered by chloroplast transformation, such as the elimination of position effects, polycistronic expression, and massive protein production, the technique represents an advance in biosafety terms; however, even if its great biotechnological potential, crops that have efficiently transformed are still a proof of concept. Despite efforts, other essential crops have remained recalcitrant to chloroplast transformation, which has limited their expansion. In this chapter, we address the most recent advances in this area and the challenges that must be solved to extend the transformation to other crops and become the de facto tool in plant biotechnology.

Keywords: chloroplast, engineering, agronomical traits, biofuels, biopharmaceuticals, mass protein production

1. Introduction

It has recently been projected that the world population will reach 9.8 billion people in the next three decades, considering that plants provide about 80% of the food that is consumed and that traditional agriculture is inefficient [1–3], meeting their food and public health needs constitutes a challenge. Therefore, seeking to meet the requirements of this growth, biotechnology has used plants as platforms for the

production of proteins, and this has been exploited by different industries such as energetic, pharmaceutical, and agricultural, which have already obtained fortified foods, vaccines, antibodies, plants that exhibit resistance to pests and diseases as well as plants with low content of cell wall components [4].

Modified plants have usually been developed by inserting genes directly into the nuclear genome because of the facilities this offers, such as the possibility of using different transformation methods, high transformation frequencies, and the fact that different plant species can be transformed using a single vector type. However, the use of chloroplast transformation seems to be increasing because, although it has lower levels of transgene integration and there are difficulties in obtaining homoplasmic plants [5], it allows the containment of transgenes, which can even be expressed in a polycistronic way, it avoids position effects, and above all, increase the production levels of recombinant proteins. In addition, an advantage that can be exploited is that chloroplasts can function as biocapsules, accumulating proteins of therapeutic interest, and these can pass through the digestive tract without disturbance [6, 7].

Although chloroplast transformation has been implemented in a broader set of plant species, it has not yet been satisfactorily implemented in important crops, such as cereals, considering that these provide about 50% of the proteins in the human diet [8], limits the expansion of this technology. However, although chloroplast transformation still has challenges, it offers an opportunity to lower the production costs of recombinant proteins without biosafety concerns. In this chapter, we explore the advances in the genetic engineering of chloroplasts to produce proteins of industrial interest and the challenges that must be overcome to establish this technology as the preferred tool in the expression of recombinant proteins.

2. Genetics of the chloroplast

Chloroplasts are small organelles primarily associated with their role in the photosynthetic process, which is possible because they can obtain electrons from water [9, 10]. However, other major metabolic processes also occurred within them, such as the biosynthesis of phytohormones, vitamins, pigments, starch, or phenols [11, 12]. Also, the biochemical processes that occur in the chloroplast largely determine the plant's adaptation to its environment [13, 14].

Even though the chloroplasts are semi-autonomous, the control of chloroplast biogenesis and the chloroplast metabolic processes is mainly under nuclear control because, from the ~3000 proteins that there are in the chloroplast, 97% of them are encoded by the nuclear genome and imported via the Toc/Tic machinery [15–17]. Nevertheless, some critical proteins are produced in the chloroplast. There are involved in the transcription, replication, and translation, as well as the proteins that make up the complexes of NADPH plastoquinone oxidoreductase, cytochrome b_6f, and the ATP-synthase subunit, as well as the photosystems I and II, envelope membrane proteins, ß-subunit of acetyl-CoA-carboxylase, and cytochrome C biogenesis [16, 18].

The proteins produced in the chloroplast are encoded by ~120 genes, which are arranged in a circular double-strand genome of quadripartite structure [16, 19, 20] of which, depending on the species and the tissue age, there are multiple copies [21], *e.g.,* 22 copies in potato leaves and 900 copies in wheat leaves. However, the reason for this is not yet known [22, 23].

It has been considered that most chloroplast genomes are highly conserved in terms of their organization and content. However, the length genome varies from

~150 to 220 kb and always appears to be associated with the contraction or expansion of the Inverted Repeat regions [11, 16, 24], which are essential to gene conservation, replication initiation, stabilization, and the evolution of the chloroplast genome [21, 25, 26]. Nevertheless, this is still under discussion because there are species of angiosperms (pea and alfalfa) and gymnosperms (pine) that lack these regions and species such as *Euglena gracilis* that have three sequences but, like repeated sequences clustered in tandem [27–29].

Chloroplast genomes are organized into nucleoids whose number, size, composition, and structural organization are varied, even between adjacent nucleoids [16]. These nucleoids are attached to the thylakoid membrane through different proteins such as MFP1, TCP34, and pTAC16, and seem to be involved in the regulation of gene expression through their association with different SWIB-domain proteins, CND41, sulfite reductase, and WHIRLY proteins [30–33]. In addition, there is strong evidence that nucleoids serve as platforms for forming ribosomes [34, 35]. Therefore, it is currently accepted that nucleoids participate in replication, transcription, translation, post-translational regulation of gene expression, and repair of chloroplast genomes.

Although the number of nucleoids and genomes per nucleoid is variable, it is considered that a nucleoid comprises 10 to 20 copies of plastid DNA. The large number of chloroplast genomes with the possibility of polycistronic expression has made chloroplast genetic engineering rapidly growing [36, 37].

3. Incorporation of new traits in plants through chloroplast transformation

The growing demand for agricultural products has influenced the search for alternatives that maximize their production, including developing modified plants [38]. These crops have allowed an increase in agricultural production, which has also translated into higher profits for farmers [39, 40], to such a degree that by 2018, the commercialization/planting of modified plants had already been adopted by 71 countries [41], which is a rapid growth rate given that it was introduced to the market in 1995, just 23 years prior. The incorporation of new characteristics to plants has maintained an interest in modified crops, so much so that to date, 88% of cotton and 82% of corn grown in the United States is genetically modified [42], and this trend continues, in March 2023 Brazil approved the wheat-HB4 (*Triticum aestivum*) crop that exhibits the *Hahb-4* transcription factor from *Helianthus annuus* that confers drought stress tolerance, as reported by the International Service for the Acquisition of Agri-biotech Applications (event: IND-ØØ412–7) [43].

The development of the first modified plants was achieved by nuclear transformation with the intention of conferring resistance to biotic and abiotic stresses [44–46], and although this method of improvement has been maintained the leadership of plant genetic engineering, to date, we are still grappling with problems such as gene silencing, random transgene integration, inappropriate gene expression regulation, genomic instability, interference with other genes, and selection issues. These problems can affect the expression and function of the transgene, as well as its stability and heritability. Also, there is an ecological risk of the crop-to-crop gene flow [47–49].

To reduce the problems associated with nuclear transformation, biotechnology has gone deeper into the genetic engineering of chloroplasts, which considerably improves the expression levels of recombinant proteins and decreases the unwanted effects associated with modified crops.

The genetic engineering of chloroplasts seems to be growing rapidly, which is possible thanks to the standardization of DNA delivery protocols, the understanding of the mechanisms that govern transformation efficiency, as well as the increase in sequenced chloroplast genomes, which have gone from 6768 in 2021 to 10,712 reported in the RefSeq database from the National Center for Biotechnology Information (NCBI) today, and of which 1072 of them have been reported only so far in 2023.

Although establishing chloroplast transformation in different crops has been challenging, it has already been successfully established in different species (**Table 1**), and currently, been developed plants that express enzymes of industrial value, antigenic

		Family	Species	Ref.
Plants	*Dicots*	Solanaceae	*Nicotiana sylvestris, Nicotiana tabacum* var. Petit Havana, and *Nicotiana benthamiana*	[50–53]
			Petunia hybrida var. Pink Wave	[54]
			Solanum melongena	[55]
			Solanum lycopersicum	[56]
			Capsicum annuum var. G4	[57]
			Solanum tuberosum cv. Desirée and line 1607	[58, 59]
		Brassicaceae	*Brassica napus* cv. FY-4	[60]
			Brassica oleracea var. capitata	[61]
			Brassica oleracea var. botrytis	[62]
			Lesquerella fendleri	[63]
			Brassica napus	[64]
			Arabidopsis thaliana	[65]
		Fabaceae	*Medicago sativa* cv. Longmu 803	[66]
			Glycine max	[67]
		Apiaceae	*Daucus carota* cv. Half long	[68]
		Malvaceae	*Gossypium hirsutum* cv. Coker310FR	[69]
		Asteraceae	*Artemisia annua*	[70]
			Lactuca sativa cv. Verônica, cv Flora and cv. Cisco	[71–73]
		Plantaginaceae	*Scoparia dulcis*	[74]
		Cucurbitaceae	*Momordica charantia*	[75]
		Amaranthaceae	*Beta vulgaris*	[76]
		Salicaceae	*Populus alba*	[77, 78]
	Monocots	Poaceae	*Saccharum officinarum*	[79]
			Oryza sativa var. Japonica line 19 and Hwa-Chung	[80, 81]
Liverworts		Marchantiaceae	*Marchantia polymorpha*	[82]
Moss		Funariaceae	*Physcomitrella patens*	[83]
Algae	*Microalgae*	Isochrysidaceae	*Tisochrysis lutea*	[84]

	Family	Species	Ref.
	Monodopsidaceae	*Nannochloropsis oceanica*	[85]
	Phaeodactylaceae	*Phaeodactylum tricornutum*	[86]
Red algae	Cyanidiaceae	*Cyanidioschizon merolae*	[87]
	Bangiaceae	*Pyropia yezoensis*	[88]
	Porphyridiophyceae	*Porphyridium* sp. UTEX 637	[89]
Green algae	Dunaliellaceae	*Dunaliella tertiolecta*	[90]
	Haematococcaceae	*Haematococcus pluvialis*	[91]
	Euglenaceae	*Euglena gracilis*	[92]
	Chlamydomonadaceae	*Chlamydomonas reindhartii*	[93]

Table 1.
Species transformed by stable chloroplast transformation.

proteins for vaccine production, proteins of pharmaceutical interest, and proteins for the production of biofuels and biomaterials [36, 94–97], as well as proteins that confer resistance to pests and diseases [77, 98, 99].

3.1 Chloroplast transformation to confer resistance to pests

Annually, about 40% of agricultural products are lost before harvest due to attacks by insects, weeds, and plant pathogens, increasing by 20% after harvest [100]. Therefore, using pesticides has been one of the alternatives that have allowed controlling these losses, increasing their use in cropland from 1.55 kg Ha^{-1} in 1990 to 2.69 kg Ha^{-1} in 2019 [101].

The use of pesticides has contributed significantly to agricultural production. However, the emergence of pests resistant to these chemicals (>17,000 cases of resistance amongst 612 species globally by 2020) [102] has resulted in a greater reliance on higher doses and the introduction of new pesticides, which is a worrying trend since it can lead to environmental contamination, and more significant risks for human health; by the way, each year, millions of people around the world experience unintentional acute poisoning by pesticides (~385 million reported cases), of which 11,000 cases end in death [103]. Although this figure is already worrying in itself, it must be considered that 50% of pesticides are organophosphates. These are of particular concern since they pass from the roots to the leaves and can be consumed by the public, which increases the risk of poisoning [104–106].

Pursuing sustainable and effective alternatives to protect crops without the negative consequences of excessive pesticide use resulted in the introduction of the first insecticidal gene, Cry1Ab, to plants through nuclear transformation [44]. Since then, numerous studies have demonstrated the efficacy of Cry proteins against various pests, including *Plutella xylostella Sesamia inferens*, *Chilo suppressalis*, *Herpitogramma licarisalis*, *Mycalesis gotama*, *Cnaphalocrocis medinalis*, *Scirpophaga incertulas*, *Naranga anescens*, *Parnara guttata,* and *Elasmopalpus lignosellus* [107–111]. Cry proteins have also been tested to control nematodes, Phthiraptera, Orthoptera, mites, Coleoptera, Lepidoptera, Diptera, Hymenoptera, Hemiptera, and protozoa [112, 113]. The usefulness of these proteins has led to their continued study and exploration, with over 700

insecticidal proteins already reported (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). However, insects' resistance to Bt crops has been reported (from 3 cases in 2005 to 16 in 2016), affecting crops containing Cry1Ab, Cry1Ac, Cry1A.105, Cry1Fa, Cry2Ab, Cry3Bb, mCry3A, eCry3.1Ab, and Cry34/35Ab [114, 115]. On the other hand, there is a direct observation of resistance by *Diabrotica virgifera virgifera* to the eCry3.1Ab protein in the field before the plants were commercialized [116, 117]; such resistance may increase through cross-resistance.

Although to reduce the risks of insect resistance to Bt crops have been used strategies such as multiple toxins, protein engineering, ultra-high doses, and refugees [118], the existence of other risks of the nuclear transformation, such as ecological risks and collateral effects in the expression of the genes, have motivated the use of chloroplast engineering for the expression of proteins with insecticidal potential, which began with the expression of the Cry1Ac protein in tobacco chloroplast obtaining protection against lepidopteran *Heliothis virescens, Helicoverpa zea* and *Spodoptera exigua* [45].

Other Cry proteins such as Cry9Aa2, Cry1Ia5, Cry2Aa2, Cry1C, and Cry1Ab have also been expressed in chloroplast from soybean, cabbage, and poplar, including protecting the plants against *Phthorimaea operculella, Hyphantria cunea, P. xylostella, Lymantria dispar, Anticarsia gemmatalis* and *Helicoverpa armigera* [77, 119–123], and until today, the insects did not show resistance to Cry proteins compartmentalized in the chloroplast; therefore, the accumulation proteins in the chloroplast, either through their direct expression within it or by redirecting nuclear-expressed proteins to the chloroplast for storage, seems to be a viable option to avoid the development of resistance. Although this last option it as already been analyzed with the expression of Tvip3A*, Cry1Ac, Cry1Ah, and Cry2A proteins [124–127], and is very attractive because it benefits from the facilities offered by nuclear transformation, the compartmentalization of proteins does not eliminate the risks of environmental contamination, nor any other undesired effect caused by gene insertion into the nuclear genome. Therefore it requires a more detailed analysis.

Other proteins with insecticidal activity have been expressed using chloroplasts, such as a chloroperoxidase from *Pseudomonas pyrrocinia* and the *pta* gene from *Pinellia ternata* agglutinin being effective in the control of *Alternaria alternata, Aspergillus flavus, Escherichia coli, Fusarium moniliforme, Pseudomonas syringae, Verticillium dahliae, Colletotrichum destructivum*, and *Fusarium verticillioides* [128–130].

Although the Cry proteins with which Bt crops have been nuclearly armed have shown excellent performance in controlling different pests, the expression of lectins and other proteins with insecticidal capacity from *Bacillus toyonensis* and *Lysinibacillus sphaericus* has also been shown to be effective in controlling pests such as *Alphitobius diaperinus, Spodoptera exigua, Cydia pomonella, Anthonomus grandis, Aedes aegypti*, and *Myzus persicae* [131, 132]. Furthermore, while the expression of these proteins in chloroplasts has not been thoroughly investigated, it is important to note them because despite the efficacy of Bt crops in controlling Lepidoptera and Coleoptera, control of Hemiptera has not been entirely successful, as many Hemipteran species have now become significant pests of Bt crops [133, 134].

3.1.1 Expression of RNAi for pest control

In recent years, it has been proposed that the expression of interference RNA (RNAi) for pest control in the chloroplast is a promising alternative to protein expression, and although the RNAi has been achieved previously by nuclear transformation using both double-stranded RNA (dsRNA) and long hairpin RNA (hpRNA)

[135–137], the obtained level protection in plants has been insufficient for practical application [138].

One problem in achieving high levels of protection has been the impossibility of accumulating large amounts of unprocessed RNAi in the host because the nuclear-expressed RNAi are processed by the cellular machinery leaving little raw RNAi available for ingestion by the host [139, 140]. Furthermore, when RNAi is ingested, the host's digestive system degrades another part of the ingested RNAi, resulting in a reduced amount of RNAi available for effective interference [141]. Therefore, the chloroplast has been visualized as a viable solution because it offers three crucial advantages 1) there is no RNAi processing machinery in these organelles, 2) they can produce and accumulate large amounts of RNAi [142–144], and 3) the chloroplast acts as a natural bioencapsulation method to protect the RNAi when consumed by insects [145, 146].

There is limited information on RNAi expression in the chloroplast, but the published reports show an area of high potential. For example, recently [138] expressed dsRNA targeting the *MpDhc64C* gene, a newly identified target gene whose silencing causes the lethality of the green peach aphid *Myzus persicae*. Results revealed that transplastomic plants exhibited significant resistance to aphids, which in turn showed reduced survival, decreased fecundity, and decreased weight of survivors, a similar effect to the obtained by Ren, Cao [147] with the use of dsRNA β-*Actin to control of the beetle Henosepilachna vigintioctopunctata in transplastomic potato plants.*

Other RNAi studies in chloroplasts have shown results that go the same way. In 2015, Jin et al. [141] silenced the V-ATPase, chitin synthase (*Chi* gene), and cytochrome P450 monooxygenase (*P450* gene) from *H. armigera* using dsRNA, reducing the weight and growth of larvae. Also, a hpRNA targeting acetylcholinesterase (*ACE* gene) of *H. armigera* and a dsRNA targeting β-actin and Vps32 (*ACT* and *SHR* genes, respectively) from Colorado potato beetle provided substantial protection against *H. armigera* and reduced growth of larvae of Colorado potato beetle in transplastomic tobacco and potato plants [140, 143].

Although the RNAi expression in chloroplast has been successfully established with promising results of accumulation and stability, future studies should be focused on elucidating the ideal length of the RNAi since there are reports of dsRNA with a length of 200 nt more protective than a dsRNA of 60 nt or > 200 nt; also, it has been reported that the cellular machinery can degrade the long RNAi producing siRNA that have a less insecticidal effect [136, 140]. Therefore, the adequate length of the RNAi that must be expressed is still not entirely clear, and this is important because the length and type of RNAi, whether it is hpRNA or dsRNA, dramatically influences the accumulation [138, 142, 144].

Other aspects that should also be considered are the suitable target genes and the implementation of efficient RNAi delivery methods, even though topical RNAi has recently been reported and seems a cost-effective alternative [148]. Currently, micro-injection and overall ingestion are the most used. Moreover, this last, in practice, should be carefully considered because there are pests that do not have direct contact with chloroplasts and considerably impair RNAi efficacy [149].

In 2017, was approved the first variety of transgenic maize Smartstax PRO®, which nuclearly express a dsRNA of the *Snf7* (Sucrose non-fermenting 7) gene from *D. virgifera virgifera* [150–152], whose commercial launch was in 2022, which supports the idea that RNAi technology has great potential, and although there is no product developed from chloroplast transformation on the market yet, RNAi technology may help chloroplast engineering in this process.

3.2 Chloroplast transformation to confer abiotic stress tolerance

Reactive oxygen species (ROS) are forms of oxygen partially reduced and produced during biotic and abiotic stress. Although ROS are commonly associated with stress, they are also produced under cellular respiration and photosynthetic processes [153]. Nevertheless, although ROS are produced in normal metabolic processes, the uncontrolled production of ROS causes an alteration of the correct function of the cells. Therefore, decreasing ROS in plants is still an essential target in biotechnology.

Nuclearly have been expressed genes to promote the tolerance to different abiotic stresses such as cold stress (*CsWRKY46* gene) [154], oxidative stress (*ScVTC2* gene) [155], drought and salt stress tolerance (*ZmSNAC13* and *ZmWRKY86* gene, respectively) [156, 157], metal tolerance (*OsMYB-R1* and *SbMT-2* gene) [158–160], ROS decrease (*CfAPX* gene) [160] and tolerance to waterlogging (*HaOXR2* gene) [161] and have been obtained satisfactory results; nevertheless, trying to improve the results obtained by nuclear expression, genes have been expressed in chloroplasts aimed at increasing the antioxidant pathway, intervening in the glycine betaine (GB) pathway such as *codA* gene from *Arthrobacter globiformis* [98, 162, 163].

Other proteins have also been expressed in the chloroplast, such as flavodoxin (*fld* gene) [164], arabitol dehydrogenase (*ArDH*) [165], *otsB-A* operon (trehalose phosphate synthase/phosphatase) [166], γ-tocopherol methyltransferase (*TMT* gene) [167], betaine aldehyde dehydrogenase (*badh* gene) [68], dehydroascorbate reductase (*DHAR* gene), superoxide dismutase (*MnSOD* gene), glutathione reductase (*gor* gene) [168], glutathione-S-transferase (*GST* gene) [162, 169], homogentisate phytyltransferase (*HPT* gene), conferring tolerance to salt, cold, UV-B radiation, heavy metal, and osmotolerance.

Despite the potential and benefits of plastid transformation in protecting plants, it is challenging to confer abiotic stress resistance due to the involvement of various metabolic pathways. Therefore, providing a robust resistance would require multiple gene expressions. These must be carefully selected since the expression of certain types of genes can cause pleiotropic effects because the encoded proteins could interfere with the structure and function of the thylakoids or decrease the levels of ATP production [170, 171]. Nevertheless, despite the challenges of achieving stress resistance, the population's constant growth requires finding strategies to address this need.

3.3 Expression of hydrolytic enzymes in chloroplasts

Lignocellulose residues are the most abundant raw material and a highly renewable carbon source on earth [172], and due to particularities as its abundance, availability, and sustainability is believed to be a solution to solve fossil fuel shortage [173–175], to such a degree that global ethanol and biodiesel production is projected to rise to 132 billion liters and 50 billion liters, respectively, by 2030 [176]. However, although lignocellulosic compounds are abundant [177, 178], a large part of this renewable energy is beyond our reach; that is, despite being produced each year more than 40 million tons of non-edible plant material, lignocellulose is not the most important feedstock for biofuel production because it is not efficiently processed [176, 179].

The processing problem is caused by the presence of lignin that imbibes both the cellulose as well as hemicellulose and limits its degradation [180–182]. Therefore, physical and chemical methods have been used to increase biomass degradation.

However, apart from the fact that these methods can represent a potential ecological risk, they increase production costs.

In the search for alternatives to improve biomass degradation, the use of multiple enzymes has been recurring, and in this sense, different organisms have been the source of them [183]; nevertheless, the hydrophobic nature of the substrate, the enzymes cost, the concentration of required enzymes, as well as the release of phenolic compounds during the enzymatic reaction such as xylan that can inhibit enzyme activity, have limited their use [184–186], forcing the search for new and more efficient enzymes as well as an efficient method for their accumulation; therefore, the protein expression in the chloroplast it has presented as a promissory strategy to aboard the challenges of the degradation of lignocellulosic biomass.

The expression of enzymes capable of degrading cell wall polymers into chloroplast has already been tested successfully. However, although different genes of pectinases, manganese peroxidase, cutinase, and laccase enzymes from *Fusarium solani*, *Streptomyces thermocarboxydus*, *Phanerochaete chrysosporium*, *Trichoderma reesei*, and *Pleurotus ostreatus* has been tested with promissory results [187–190], cellulases have been the subject of the most intensive search because they represent 40% of plant biomass [177, 178].

Different cellulase enzymes have already been expressed in the chloroplastic compartment with satisfactory results, *e.g.,* xylanases (*xynA, xyl, xyn2, Xyl10B, xynA, xyn10A,* and *xyn11B* genes) from *Bacillus subtilis, Trichoderma reesei, Clostridium cellulovorans, Thermotoga maritima, Clostridium cellulovorans,* and *Alicyclobacillus acidocaldarius* [191–194], as well as endo-, exo-glucanases, and β-glucosidases (*cel6A, cel9A, cel6B, Cel6, Cel7, EndoV, CelK1, Cel3, TF6A, Bgl-1, bgl1C, EGPh, celA,* and *celB* genes) from *Trichoderma reesei, Thermomonospora fusca, Pyrococcus horikoshii, Chaetomium globosum, Paenibacillus* sp., *Phanerochaete chrysosporium*, *Aspergillus niger, and Thermotoga neapolitana* [36, 195–200]. Despite the expressed proteins being functional, it should be considered that the microorganisms that possess efficient mechanisms for cellulose degradation have redundant genes often [201]. Therefore, high degradation depends not on a particular protein but on its enzymatic cocktail, which should be considered to improve enzymatic processes.

Regardless of the intense search for cell wall hydrolytic enzymes that have been carried out, there is still a need to identify efficient cellulose enzymes [184] because they are critical factors in paper recycling, cotton processing, juice extraction, detergent production, food industry, animal feed additives and largely determine the price of biofuels [202–204]. For this reason, it is necessary to use strategies that reduce cellulase enzyme production costs as much as possible to make them commercially viable on a larger scale. Further studies will continue to be carried out to express cellulases in chloroplasts.

3.4 Chloroplast engineering for the biopharmaceutical industry

Population growth implies a constant demand for medicines, representing a lucrative opportunity for the pharmaceutical industry, which only in 2022 billed ~1.48 trillion U.S. dollars, an increase of 4.23% concerning 2021. However, most of the world's population cannot access medicines due to unaffordable prices [205–207]. Therefore, products with nutritional and pharmaceutical value are gaining importance to such an extent that there are currently 1775 products with different formulations, biosimilars, and biobetters approved by the Foods and Drugs Administration (FDA) and European Medicines Agency (EMA) for use in humans [208], which can reduce the costs of medical treatments.

Chloroplasts have already begun to contribute to the production of plasma proteins, vaccines, antibodies, and enzymes [97, 209, 210], which is especially relevant since two of the current challenges in treatments with proteins for oral consumption are obtaining high protein concentrations and the possible degradation that they may experience when passing through the digestive system [206]. These challenges could be overcome by the accumulation and bioencapsulation of proteins in chloroplasts [211].

Two decades have passed since the first candidate antigen against a human disease was expressed [212], and since then, other proteins have been expressed, *e.g.,* A27L immunogenic protein (*A27L* gene) [213], ESAT6, Mtb72F [214, 215], *Angiotensin-converting enzyme 2 (ACE2* gene), Angiotensin (1–7) (*Ang-*[*1–7*] gene) [7, 211], Coagulation factor VIII (*F8* gene) [216], E7 Human papillomavirus antigen (*E7* gene) [217], Coagulation Factor IX (*F9* gene) [96], SAG1 Surface antigen (*SAG1* gene) [95], EDIII-1, EDIII-3, EDIII-4 (*ediii-1, ediii-3*, and *ediii-4* genes) [218], KETc1, KETc7, KETc12, GK1, TSOL18/HP6-Tsol [219], Griffithsin protein (*grft* gene) [220], S1D [221], and Human epidermal growth factor (*hEGF* gene) [94] in plants; and recently, was cloned the gene *IL29* in alga to produce human interleukin 29. The proteins expressed in the chloroplastic compartment have been obtained in high concentrations retaining their functionality, showing the potential value of expressing biopharmaceutical proteins in chloroplasts [222].

One of the challenges currently faced by therapeutic proteins is to stimulate their passage through the epithelial mucosa [223]; however, this challenge has been efficiently addressed by expressing the proteins in the chloroplast fused with the cholera toxin B subunit (CTB), and this has already been tested with the expression of exendin-4 (CTB-EX4) and acid alpha-glucosidase (CTB-GAA) [6, 224]. Fusing the proteins to CTB is an important approach as it has been reported that it could help promote oral tolerance; however, other non-toxic fusion proteins have been reported, such as human transferrin, although there are no reports with this fused protein in the chloroplast.

There is a need for biobetters/biosimilars on the market, and expressing therapeutic proteins in the chloroplast is not only feasible but could solve delivery and efficacy issues. To date, no one plant/chloroplast-based vaccine against human diseases is available to the public. On the other hand, although transplastomic plants have been cultivated for just over a decade with the consent of the United States Department of Agriculture Animal and Plant Health Inspection Service USDA-APHIS, they have not been scaled to the commercial level even though these plants do not fit USDA-APHIS regulation 7 CFR part 340 [206].

4. Conclusions

Given that the world population has been projected to increase by just over 20% in 30 years, it becomes evident that it will be necessary to adopt new technologies to face this situation in areas such as agriculture, energy, and pharmaceuticals, which are key sectors to guarantee well-being and sustainability in the future. For almost three decades, chloroplast transformation has shown important qualities that can help us address the challenges, offering greater efficiency and performance in the expression of modified genes and more precision and control in genetic modification accompanied by greater environmental safety and biosafety. While some challenges need to be addressed, such as the optimization of regulatory regions, limited expression of

proteins in non-photosynthetic tissues, the understanding of the mechanisms that govern pleiotropic effects, and the current inability to transform cereals efficiently, it is a fact that it can play a fundamental role in the production of products.

Conflict of interest

The authors declare no conflict of interest.

Author details

Brenda Julian Chávez[1], Stephanie Solano Ornelas[1], Quintín Rascón Cruz[1],
Carmen Daniela González Barriga[2], Sigifredo Arévalo Gallegos[1],
Blanca Flor Iglesias Figueroa[1], Luis Ignacio Siañez Estrada[1], Tania Siqueiros Cendón[1],
Sugey Ramona Sinagawa García and Edward Alexander Espinoza Sánchez[1*]

1 Biotechnology Laboratory I, Faculty of Chemical Sciences, Autonomous University of Chihuahua, Chihuahua, Mexico

2 Tissue Culture Laboratory, Division of Engineering and Sciences, Monterrey Institute of Technology and Higher Education, Chihuahua, Mexico

*Address all correspondence to: eaespinoza@uach.mx

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