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Review

Plastid transformation: Advances and challenges for its implementation in agricultural crops

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ABSTRACT

Chloroplast biotechnology has emerged as a promissory platform for the development of modified plants to express products aimed mainly at the pharmaceutical, agricultural, and energy industries. This technology's high value is due to its high capacity for the mass production of proteins. Moreover, the interest in chloroplasts has increased because of the possibility of expressing multiple genes in a single transformation event without the risk of epigenetic effects. Although this technology solves several problems caused by nuclear genetic engineering, such as turning plants into safe bio-factories, some issues must still be addressed in relation to the optimization of regulatory regions for efficient gene expression, cereal transformation, gene expression in non-green tissues, and low transformation efficiency. In this article, we provide information on the transformation of plastids and discuss the most recent achievements in chloroplast bioengineering and its impact on the biopharmaceutical and agricultural industries; we also discuss new tools that can be used to solve current challenges for their successful establishment in recalcitrant crops such as monocots.

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1. Introduction

For many years, plants' nuclear genome has been a target for genetic engineering to produce bio-factories. Despite conservatism, the modified plants have emerged as one of the most promising scalable platforms for protein production [1]. The main objective has been to produce large amounts of useful proteins that can meet the needs of a growing population projected to exceed nine billion people by 2050. Plants that produce vaccines, antibodies, fortified foods, plants with useful agronomical traits such as resistance to pests and diseases, and plants with low cell wall content components are currently in development for pharmaceutical, agricultural, and energy industries.

Nuclear transformation has made it possible to obtain modified plants that reduce the production costs of the proteins of interest; however, this also leads to biosafety problems. The transformed plants can hybridize with wild relatives, allowing transgenes to be dispersed uncontrolled [2]. These observations are conducive to the search for new strategies that can reduce or eliminate the environmental impact, and this is where chloroplast transformation makes its contribution. Perhaps from a public and ecological point of view, gene containment is the most notable advantage that plastid transformation offers. However, other advantages include the high level of protein expression produced by transplastomic plants without gene silencing, multi-gene engineering, and the ability to eliminate position effects [3,4].

At present, the study and manipulation of chloroplast DNA have become accomplished in many laboratories worldwide, having successful transformations in several species in a stable form, mainly in dicot plants. There are already well-established transformation protocols; however, despite having achieved a stable transformation in rice [5], it remains a fact that monocot crops are recalcitrant to plastid transformation. Pleiotropic effects have also been reported in some transplastomic plants [6,7], so more studies are required to make the plastid transformation more efficient.

The biosafety feature and inherent capacity for mass production of recombinant proteins offered by plastid transformation have made it a rapidly growing area; however, it still has to deal with several drawbacks to be considered successful [8]. Thus, in this review, we explore the advances in the plastid transformation, the strategies that would allow us to increase the expression of proteins of interest in plastid compartments, and the challenges that must be solved to extend this technology to other plant species.

2. Chloroplast biology: the machinery behind protein production

Chloroplasts are organelles involved in the biosynthesis of fatty acids, nitrogen and amino acid metabolism, phenol compounds, purines, pyrimidines, isoprenoids, starch, pigments, vitamins synthesis, and also are implicated in the metabolism of phytohormones such as cytokinins, abscisic acid, and gibberellins—as a result, any interruption of its normal metabolism can be lethal in plants [9].

About 3000 proteins are accumulated within it to control chloroplast metabolism, and many of them are encoded in the cell nucleus; very few are encoded in the chloroplast itself [10]. The proteins produced in the chloroplast are encoded by around 120 genes in a genome of ~150 to 220 kb with quadripartite structure where the particular feature in higher plants is the duplication of a large region (~25 kb) in an inverted orientation (IRs) [11,12,13], that provide genomic stability, thus requiring its maintenance for the evolution of chloroplast genomes [14,15,16,17]. Of the genes encoded in the chloroplast genome, around 47 genes, such as *psb, psa, ycf3, ycf4, pet, atp, ndh,* and *rbcL*, are involved in the formation of photosynthetic apparatus (PSII subunit, PSI subunit, PSI assembly, cytochrome b_6f complex, ATP-synthase subunit, and the chloroplast NADPH plastoquinone oxidoreductase complex subunit). More than 60 genes, such as *trn, rrn, rpl, rps, rpo, matK, clpP,* and *infA,* are involved in the genetic system (tRNA, rRNA, ribosomal proteins, RNA-polymerases, maturases, caseinolytic proteases, and factors of translation initiation). Some genes are not directly involved in the process of photosynthesis or the chloroplast genetic system, such as *accD* (ß-subunit of acetyl-CoA-carboxylase), *ccsA* (cytochrome C biogenesis), and *cemA* (envelope membrane protein) [11,16]. In addition, the chloroplast genome has hypothetical open reading frames, such as *ycf1* (Tic214) and *ycf2*, is presumably involved in protein import functions (for more information on *ycf1* and *ycf2,* see [18,19,20,21]).

The chloroplast genome is grouped into polyploid nucleoids whose number varies with the age of the tissue: from a few in meristematic plastids to >30 in mature chloroplasts (about 20–750 nucleoids per cell) [22]. This particularity of increasing the number of genomes per cell over time, which has a positive effect on the transgene copy number per cell, coupled with the fact that genes in the chloroplast can be expressed as an operon that allows for multigene engineering [11,23,24], are the reasons why the chloroplast genome is a target of plant biotechnology.

3. Plastid transformation: strategies to extend the technology to other crops

The chloroplast is responsible for the major metabolic processes in plant cells [9]. In recent years, 4891 chloroplast genomes from different species have been deposited in the National Center for Biotechnology Information (NCBI) database. This database has facilitated the understanding and manipulation of the genome in order to insert genes of interest that may help to solve the problems that arose with the nuclear transformation, but through a new route of recombinant protein expression, which is safer, more efficient and, above all, more controllable.

3.1. Species with stable chloroplast transformation

The plastid expression system is based on the insertion of exogenous DNA by homologous recombination to specific chloroplast genome sites [25]. Although chloroplast transformation was developed three decades ago in *Chlamydomonas reinhardtii* by Boyton et al. [26], its establishment in other interest species has been a

challenge. Currently, stable transformations have been carried out in higher plants, such as tobacco, cauliflower, tomato, Arabidopsis, lesquerella, artemisa, cabbage, rapeseed, poplar, alfalfa, potato, carrot, cotton, oilseed rape, eggplant, petunia, soybean, sugar beet, sweet broom, rice, bitter melon, lettuce, and pepper, although it has also transformed moss, liverworts, and algae (Table 1); the model species is still tobacco (Fig. 1).

Arabidopsis thaliana is a special case since its chloroplast transformation is highly challenging. *Arabidopsis* produces a nuclear homomeric acetyl-CoA-carboxylase (ACCase, *acc2* gene), which is imported to plastids with a partial function to the heteromeric ACCase encoded by the plastid *accD* gene involved in the metabolism of fatty acids; hence, when plastid translation is blocked by spectinomycin, used as a selective agent in transformed tissues, the nuclear ACCase enzyme enables a limited fatty acid biosynthesis in plastids, permitting the rampant growth of untransformed callus material, which prevents the efficient obtaining of transformed plants [27]; for that reason, it was hypothesized that the knockout of the nuclear gene *acc2* would produce tissues hypersensitive to spectinomycin because it would force the plastids to depend solely on the function of the plastid *accD* [28]. This was confirmed recently by Ruf et al. [29]. They obtained a high recovery of fertile transplastomic plants using root tissue from *A. thaliana* deficient in the *acc2* nuclear gene, overcoming the main obstacle for the *A. thaliana* chloroplast transformation. Consequently, this strategy could be extended to other plant species that are closely related to the Brassicaceae recalcitrant to the selection and regeneration of transplastomic cells.

3.2. Selection marker gene

Once the tissue has been transformed, the transformation event occurs only in a few plastids, and through selection rounds or the barnase–barstar system [30], the population of non-transformed plastids is diluted. The selection rounds are necessary because inducing regeneration from tissue derived from only one selection round could result in a chimeric shoot with both transformed and non-transformed plastids with less production of recombinant proteins, which do not always successfully transfer transgenes to progeny [31].

The experience has shown that two or three selection rounds have been sufficient to obtain homoplasmic plants [31,32]. The selection has been facilitated using portable selectable markers because they are already assembled within the expression vector [33].

Historically, the expression of the *rrn16* gene with specific mutations allowed for resistance against spectinomycin; however, the subsequent expression of the *aadA* gene (coding for aminogly-

Table 1
Species transformed by stable chloroplast transformation.

	Family	Species	Name	Selection marker	Ref.
Plants					
Dicots	Solanaceae	*Nicotiana tabacum* var. Petit Havana, *Nicotiana benthamiana* and *Nicotiana sylvestris*	Tobacco	*aadA*	[35,180,181,182]
		Solanum lycopersicum	Tomato	*aadA*	[37]
		Solanum tuberosum cv. Desirée and line 1607	Potato	*aadA*	[72,183]
		Petunia hybrida var. Pink Wave	Petunia	*aadA*	[40]
		Solanum melongena	Eggplant	*aadA*	[184]
		Capsicum annuum var. G4	Pepper	*aadA*	[185]
	Brassicaceae	*Arabidopsis thaliana*	Arabidopsis	*aadA*	[36]
		Brassica napus	Oilseed rape	*aadA*	[38]
		Lesquerella fendleri	Lesquerella	*aadA*	[31]
		Brassica oleracea var. botrytis	Cauliflower	*aadA*	[42]
		Brassica oleracea var. capitata	Cabbage	*aadA*	[186]
		Brassica napus cv. FY-4	Rapeseed	*aadA*	[187]
	Malvaceae	*Gossypium hirsutum* cv. Coker310FR	Cotton	*aphA-6*, *nptII*	[45]
	Apiaceae	*Daucus carota* cv. Half long	Carrot	*aadA*, *badh*	[39]
	Fabaceae	*Glycine max*	Soybean	*aadA*	[41]
		Medicago sativa cv. Longmu 803	Alfalfa	*aadA*	[188]
	Asteraceae	*Lactuca sativa* cv. Verônica, cv Flora and cv. Cisco	Lettuce	*aadA*	[57,189,190]
		Artemisia annua	Artemisa	*aadA*	[191]
	Salicaceae	*Populus alba*	Poplar	*aadA*	[192]
	Amaranthaceae	*Beta vulgaris*	Sugar beet	*aadA*	[193]
	Plantaginaceae	*Scoparia dulcis*	Sweet broom	*aadA*	[77]
	Cucurbitaceae	*Momordica charantia*	Bitter melon	*aadA*	[43]
Monocots	Poaceae	*Oryza sativa* var. Japonica line 19 and Hwa-Chung	Rice	*aadA*, *hpt*	[5,155]
Algae					
Green algae	Chlamydomonadaceae	*Chlamydomonas reinhardtii*	-	*aadA*	[34]
	Euglenaceae	*Euglena gracilis*	-	*aadA*	[194]
	Haematococcaceae	*Haematococcus pluvialis*	-	*aadA*	[195]
	Dunaliellaceae	*Dunaliella tertiolecta*	-	*ereB*	[51]
Red algae	Porphyridiophyceae	*Porphyridium* sp. UTEX 637	-	Mutated *ahas* (W492S)	[60]
	Bangiaceae	*Pyropia yezoensis*	-	*cat*	[196]
	Cyanidiaceae	*Cyanidioschizon merolae*	-	*cat*	[49]
Microalgae	Phaeodactylaceae	*Phaeodactylum tricornutum*	-	*cat*	[197]
	Monodopsidaceae	*Nannochloropsis oceanica*	-	*ble*	[50]
	Isochrysidaceae	*Tisochrysis lutea*	-	*bar*	[198]
Moss					
	Funariaceae	*Physcomitrella patens*	Moss	*aadA*	[199]
Liverworts					
	Marchantiaceae	*Marchantia polymorpha*	Umbrella liverwort	*aadA*	[200]

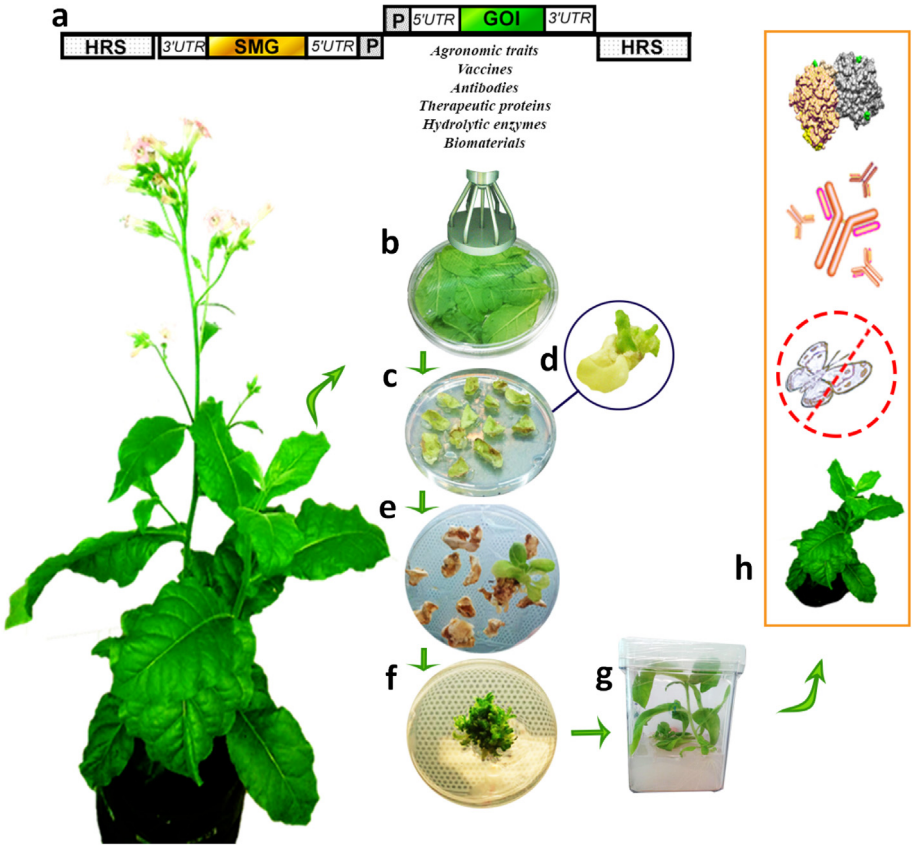

Fig. 1. Schematic representation of tobacco chloroplast transformation via biolistic. (a) Typical cassette of recombination of plastid transformation vectors which contains a gene of interest and a selection marker gene (P, promoter; 5′ and 3′UTR, untranslated region; HRS, homologous recombination site; GOI, gene of interest; SMG, selection marker gene); both, the selection marker gene and the gene of interest can be mounted on the vector to be transcribed in the same direction or placed in opposite directions. A homologous recombination site flanks the expression cassette to drive the cassette into a specific region into the chloroplast genome taken from the wild-type plastid genome. (b) The plasmid DNA is coated on gold or tungsten microparticles and then shot on sterile young leaves using a gene gun. (c) The bombarded leaves are cut and placed on regenerated media with appropriate hormones and antibiotics. (d–f) The explants are placed in two or three selection rounds to obtain homoplasmic shoots. (g and h) The regenerating shoots are transferred to the regeneration medium without antibiotics and eventually to pots. Transplastomic plants can produce antibodies, vaccines, vitamins, synthetic drugs, therapeutic proteins, fortified foods, and industrial enzymes within ~15 months, depending on the species.

coside 3″-adenylyltransferase), described by Goldschmidt-Clermont [34] in *Chlamydomonas reinhardtii*, allowed researchers to obtain resistance in the transformed shoots against spectinomycin/streptomycin. Later, the *aadA* gene was used by Svab and Maliga [35] to transform tobacco plants, obtaining up to a 100-fold frequency of transformation. These results were so satisfactory that the *aadA* gene is still used today for the transformation of various plant species *e.g.,* Arabidopsis [36], tomato [37], oilseed rape [38], carrot [39], petunia [40], soybean [41], cauliflower [42], and bitter melon [43] (Table 1).

In the transformation of plant chloroplasts, other selection marker genes have also been reportedly used for the selection of transformed cells. In plants, the following genes have been reported: *nptII* gene (neomycin) [44], *aphA-6/nptII* genes (kanamycin) [45], *hpt* gene (hygromycin) [5], *aac(6′)-Ie/aph(2″)-Ia* gene (tobramycin and gentamicin) [46], *aphA-6* gene (amikacin) [45], and *cat* gene (chloramphenicol) [47]; in algae, the following genes have been reported: *aphA-6* gene (amikacin) [48], *cat* gene (chloramphenicol) [49], *ble* gene (zeocin) [50], and *ereB* gene (erythromycin) [51].

Despite the high selection efficiency using antibiotics, they can cause tissue damage; hence, non-antibiotic selection markers have been reported. In plants, the following have been reported: betaine aldehyde dehydrogenase (*badh* gene) [52], and D-serine ammonia-lyase (*dsdA* gene) [53]; in algae, the following have been reported: cytosine deaminase (*codA* gene) [54], and phosphite oxidoreductase (*ptxD* gene) [55,56]. On the other hand, markers that are dose-dependent have been reported in plants: the mutated *ahas* gene from *Arabidopsis thaliana,* which confers tolerance to imazapyr [57], 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*) gene, which confer resistance to glyphosate [58], and the *bar* gene that confers resistance to phosphinothricin [59]; for algae, the following have been reported: the mutated acetohydroxyacid synthase gene [AHAS(W492S)], which confers tolerance to sulfometuron methyl [60]. However, the use of herbicides for selection is known to have a detrimental effect on the recovery of transformed cells [61].

Although the use of selection genes is mandatory for the recovery of transformed cells, the conservation of these genes is no longer necessary after selection. Also, there is a risk of transfer to the environment [62]. Recently, the idea of using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) to design chloroplast genomes and thereby, the possibility of eliminating genes of resistance, has been proposed [63]. Yet, the specific bombardment of plastid with CRISPR/Cas9 binary vector or its counterpart Cas9/gRNA ribonucleoproteins cannot be controlled. The impermeability of plastids to most RNA and DNA limits CRISPR/Cas9 use in the plastid [64]. Nonetheless, the results obtained by Yoo et al. [65] in *Chlamydomonas* chloroplasts open the door to engineering organelle genomes. They demonstrated, for the first time, the editing of

the chloroplast genome with Cas9 to integrate DNA with high precision in the plastome; although these results are highly promising, plastid editing is still underexplored [63], and the selection of marker genes must still be eliminated using the *Cre/loxP* system [66,67], *phiC31* phage site-specific integrase [68], and the directly repeated DNA sequences [62,69].

3.3. Regulatory sequences

The main objective of plastid transformation technology is to obtain high levels of protein expression and, because of this, it has sought to increase the abundance of mRNA. For such a purpose, different promoters (P) have been used to drive the genes of interest. A promoter with high efficiency to drive the gene expression is the *rrn* promoter (*rrn* gene), which is more than 12 times more efficient than the *psbA* or *trc* promoters in tobacco chloroplast [70], so much so that the *rrn* promoter is widely used today in plastid transformation. Other promoters have also been used in plastid transformations, such as P*T7* [71], P*clpP* [72], P*atpI* [73], P*rbcL* [74], P*accD* [75,76], and the synthetic promoter P*NG1014a* [77]. Recently, a complete modular cloning system was developed specifically for chloroplast transformation, containing 47 characterized and uncharacterized chloroplast promoters and modified versions of endogenous promoters. This constitutes a valuable and flexible tool for plastid metabolic engineering in plants [78].

To stabilize the mRNA and propitiate the mRNA accumulation, different 5′ and 3′ untranslated regions (5′ and 3′ UTRs) have been tested, such as 5′UTR from *T7g10, rbcL, atpB, psbA, psbN, ndhF* genes [72,79,80,81,82,83], as well as 3′UTR from *rbcL*, *rps16*, *psbA*, *rrnB, petD, rpoA* genes [72,73,79,80,84]. The use of these regions is important because they are recognized by proteins that protect the mRNA against exonucleases, so the loss of these regulatory elements leads to the fast degradation of transcripts.

Even though promoters and 5′ and 3′UTR could be optimized, the accumulation level of recombinant proteins in plastids cannot be anticipated because the abundance of chloroplast proteins is mainly controlled at the post-transcriptional level [85]. Another limitation is that protein accumulation could be affected by protein degradation caused by the housekeeping machinery in response to protein misfolding. This could be avoided by sending the recombinant proteins into the thylakoid lumen using transit peptides such as the 23-kDa protein of the oxygen-evolving system of photosystem II (*Str*) because, in thylakoids, proteins are not susceptible to degradation as in stroma, which improves their accumulation—see the report by Morgenfeld et al. [7,86].

4. Chloroplast engineering for the Biopharmaceutical industry

The use of plants to produce chemical compounds with nutritional and pharmaceutical interest has always been a goal of biotechnology, so much so that, from 2016 to 2020, there was a growth of 18% in products (1357–1666 products) approved by the Foods and Drugs Administration (FDA) and European Medicines Agency (EMA) for use in humans, including products with different formulations, biosimilars, and biobetters [87]. The chloroplast has already begun to contribute, expressing vaccines, enzymes, antibodies, and plasma proteins.

Nineteen years have passed since Daniell et al. [88] expressed the first candidate antigen to vaccinate against a human disease in tobacco chloroplast. Later, other proteins, such as 2L21 peptide against virulent canine parvovirus [89] and Human Serum Albumin (*HAS*) [90], were also expressed in tobacco, showing the potential of chloroplasts in the biopharmaceutical area, so much so that by 2009, there were already 23 antigens expressed against 16 different organisms [91].

In the protein expression in plastids, the cholera toxin B subunit (CTB) fused with proteins of interest has been used as an effective transmucosal carrier and immunogen that elicits mucosal antibody responses and oral tolerance [89,92,93]. Currently, several CTB-fused proteins have been reported, such as exendin-4 (CTB-EX4), whose product increased insulin secretion in pancreatic cells from mouse (beta-TC6) [94], angiotensin-converting enzyme 2 and Angiotensin-(1–7) (CTB-ACE2 and CTB-Ang-(1–7)), tested against pulmonary hypertension (PH) and ocular inflammation [95,96], myelin basic protein (CTB-MBP) against Alzheimer's disease [97], coagulation factor VIII-CTB against hemophilia A [98], acid alpha-glucosidase (CTB-GAA) against Pompe disease [99], coagulation factor IX-CTB against hemophilia B [100], the 6 kDa early secretory antigenic target (CTB-ESAT-6), and the a fusion polyprotein Mtb72F (CTB-Mtb72F) against tuberculosis [101] (Table 2). It is worth noting that CTB seems to promote protein accumulation, which has been demonstrated from the low levels of ESAT-6 non-fused to CTB reported by Saba et al. [71]. In this respect, Human transferrin (*hTf*) was reported as a fusion protein in nuclear transformation [102]; however, no protein currently expressed in plastids has used this fusion protein. Human transferrin could be used in chloroplast transformation for improving the therapeutic efficacy of biopharmaceutical proteins since transferrin is non-toxic. There are transferrin receptors in the epithelium and proliferating human cells [102,103], promoting the uptake of therapeutic proteins.

Recently, Rosales-Mendoza et al. [104] reported the efficient and simultaneous expression of KETc1, KETc7, KETc12, GK1, and TSOL18/HP6-Tsol antigens against cysticercosis and Morgenfeld et al. [7] reported the expression of human epidermal growth factor (*hEGF*) in tobacco chloroplasts. To date, not a single plant/chloroplast-based vaccine against human diseases is on the market. The only one approved by the U.S. Food and Drug Administration has been the human glucocerebrosidase made in carrot cells to treat Gaucher's Disease using a nuclear expression system [105].

The results obtained so far with recombinant proteins in plastid have been promising. The fact that the plant cell walls protect the proteins expressed in the chloroplast, like a biocapsule, gives it enormous potential [106]. However, the absence of glycosylation in the plastids could be a limitation to the production of biopharmaceutical proteins that require this modification in order to be active. Some proteins do not require glycosylation, and it is possible to work with those proteins; hence, plastid transformation efficacy could increase in subsequent years.

5. Chloroplast engineering for agronomic traits

The insertion of genes in plastids covers different areas of interest; we have been attracted to the possibility of eliminating ecological problems currently associated with nuclear-modified plants and the overexpression of recombinant proteins. Several works have been reported on chloroplast transformation focused on plant protection against biotic and abiotic stress, as well as on improving the nutritional quality of crops, which has marked the way forward in this field.

5.1. Pest and disease resistance

Insect resistance mediated by chloroplast expression dates back to 1995, with the production of Cry1Ac, an extremely toxic protein to larvae of *Heliothis virescens, Helicoverpa zea,* and *Spodoptera exigua* by McBride et al. (1995) [107]. Since then, there have been promising studies that attempt to obtain a high accumulation of insecticidal proteins expressed in the plastid, at least of the Cry proteins (Cry2Aa2, Cry1Ia5, Cry9Aa2, Cry1Ab) to protect against

Table 2
Plastid transformation, advances in biopharmaceutical products.

Gene	Product	Selection rounds	Promoter	5′UTR	3′UTR	Chloroplast region	Selection marker	Total soluble protein (TSP)	Host	Ref.
HSA	Human Serum Albumin	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	11.1%	Tobacco	[90]
2L21-CTB	Parvovirus 2L21 epitope-CTB	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	31.1%	Tobacco	[89]
gag	$Pr55^{gag}$	2	*rrn*	*rbcL*	*rbcL*	*rps12/7-*	*aadA*	6.75%	Tobacco	[170]
		2	*rrn*	*T7g10*	*rbcL*	*trnV*	*aadA*	0.26%		
A27L	A27L immunogenic protein	2	*rrn*	*T7g10*	*rbcL*	*rps12/7-trnV*	*aadA*	18%	Tobacco	[172]
EX4	Exendin-4	*	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	14.3%	Tobacco	[94]
ESAT-6	ESAT-6 protein-Mtb72F	*	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	7.5%	Tobacco	[101]
								1.2%		
								0.75%	Lettuce	
ACE2	Angiotensin-converting enzyme 2	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	2.14%	Tobacco	[96 95]
Ang-(1–7)	Angiotensin (1–7)	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	8.7%	Tobacco	[9695]
MBP	Myelin basic protein	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	2%	Tobacco	[97]
F8	Coagulation factor VIII	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	4.2%	Tobacco	[98]
E7	E7 Human papillomavirus antigen	3	*psbA*	*psbA*	*rps*	*rrn16-trnI*	*aadA*	~8%	Tobacco	[86]
GAA	Alpha glucosidase	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	0.21%	Tobacco	[99]
F9	Coagulation Factor IX	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	0.63%	Lettuce	[100]
SAG1	SAG1 Surface antigen	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	2.5%	Tobacco	[201]
*	KETc1	3	*rrn*	*T7g10*	*rbcL*	*trnN-trnR*	*aadA*	0.1%	Tobacco	[104]
	KETc7							~0.14%		
	KETc12							0.4%		
	GK1							0.45%		
	TSOL18/HP6-Tsol							0.54%		
ESAT-6	ESAT-6 protein	3	*T7*	*T7g10*	*T7*	*trnN-trnR*	*aadA*	1.2%	Tobacco	[71]
hEGF	Human epidermal growth factor	3	*psbA*	*psbA*	*rps*	*rrn16-trnI*	*aadA*	*a*	Tobacco	[7]

a Indicates observed adequate gene expression, but no quantification.
* Indicates not reported.
FW: Fresh weight

Phthorimaea operculella, Plutella xylostella, Helicoverpa armigera, and *Anticarsia gemmatalis* [108,109,110,111]. Wu et al. [112] reported the first expression of the *cry1C* gene in poplar, showing high toxicity to *Hyphantria cunea* and *Lymantria dispar*.

The Cry proteins seem to have taken the lead in 'green insecticides'. Nevertheless, nuclear transformation has shown that insects can develop resistance to cytoplasmic Cry proteins [113]; however, compartmentalizing the Cry proteins in the chloroplast could be an option to increase the accumulation levels of these proteins and thus prevent insect resistance. In this respect, directing the proteins towards the chloroplast using transit peptides has been proposed, *e.g.*, Cry1Ac, Cry1Ah, and Tvip3A* proteins [114,115,116].

Other insecticidal proteins expressed in the chloroplast have been tested, such as MSI-99, a magainin-type antimicrobial peptide reported by DeGray et al. [117], which was effective against *Pseudomonas syringae*, *Colletotrichum destructivum, Verticillium dahlia, Fusarium moniliforme,* and *Aspergillus*. Later, Wang et al. [118] reported the MSI-99 effectiveness against *Alternaria alternata* and *E. coli. Pinellia ternata* agglutinin (*pta* gene) has also been shown to be effective against *Bemisia tabaci, Myzus persicae, Helicoverpa zea, Heliothis virescens, Spodoptera exigua, Erwinia carotovora,* and tobacco mosaic virus [119]. The chloroperoxidase (*cpo* gene) from *Pseudomonas pyrrocinia* also was reported to protect against *Aspergillus flavus, Fusarium verticillioides, Verticillium dahlia,* and *Alternaria alternata* [120]. Although the proteins mentioned above were efficiently expressed in tobacco chloroplasts, they still need to be explored in more detail to avoid the detrimental effect that overexpression of some of these proteins on the plant phenotype [118].

5.1.1. RNAi against pests and diseases

Until recently, the plastid transformation to confer resistance to pests and diseases has focused on expressing specific proteins, promoting their accumulation in the plants. However, the expression of interference RNA (RNAi) in plants to silence specific essential genes within herbivorous pest (trans-kingdom RNA interference, TK-RNAi) has gained momentum because it eliminates protein dependency the possible problems associated with them in both plants phenotype and ecosystem.

The expression of RNAi in plants to silence specific genes has already been tested in an effective form through nuclear expression using double-stranded RNAs (dsRNA) and long hairpin RNA (hpRNA) [121,122,123]. One of the challenges for RNAi technology's efficiency is to ensure viable delivery in insects. Due to this difficulty, oral administration is one of the most attractive methods in practice [124,125]. The host's digestive system degrades the RNAi, so high RNA concentrations are required in the artificial diet [126], which is another challenge by itself, because, with nuclear expression, the accumulation of RNAi is affected by the cell's RNAi machinery, which decreases the amount of unprocessed dsRNA or hpRNA available to pest ingestion [127,128].

The most viable solution to ensure the accumulation and stability of the RNAi would be to use the chloroplast as a natural method of bioencapsulation to protect them. In addition, the chloroplast has almost no RNAi processing machinery, if any, there is no transference of RNAi from the chloroplast to the cytoplasm [106].

The efficient expression of RNAi in chloroplast has already been reported by Jin et al. [126] in tobacco using a dsRNAs to silence chitin synthase (*Chi* gene), cytochrome P450 monooxygenase (*P450* gene), and V-ATPase from *Helicoverpa armigera,* reducing the weight and growth of larvae. dsRNA was also used by Zhang et al. [128] in potato chloroplast to silence the β-actin (*ACT* gene) and Vps32 (*SHR* gene) from Colorado potato beetle, resulting in reduced larval growth. On the other hand, the dicing of dsRNA into siRNA by the plant's intrinsic RNAi machinery may reduce dsRNA's pesticidal activity. In order to avoid this, Bally et al. [106] used hpRNA (~200 nt) targeting the acetylcholinesterase (*ACE* gene) of *Helicoverpa armigera* in tobacco chloroplast, obtaining full-length hpRNAs with strong protection against *H. armigera* herbivory. In this sense, it has been reported that dsRNA with a length of 200

nt is more protective than a dsRNA of 60 nt or >200 nt [129]. There also appears to be a negative correlation between the dsRNA's length and accumulation [129,130].

Although the chloroplast produces and stores large amounts of dsRNA and hpRNA, the RNAi presents contradictory studies, *e.g.,* Dong et al. [131] reported more effective insecticidal activity with RNAi produced by nuclear expression than by expression in tobacco chloroplast. Despite the difficulties that have arisen in this field, the RNAi can be an important tool in plant biotechnology, and dsRNA produced in chloroplast could be a significant area of opportunity because this field of research would impact the agronomic sector and could also be used to down-regulate genes associated with human diseases.

5.2. Abiotic stress tolerance

Reactive oxygen species (ROS) are forms of oxygen that are partially reduced and routinely produced during normal metabolic processes such as photosynthesis or cellular respiration, and under biotic and abiotic stress. Due to the light-dependent electron transport processes, the chloroplast is an essential ROS factory because they have a high capacity for the oxidation of lipids membranes, nucleic acids, and proteins, causing cell damage [132].

Decreasing ROS in plants has been a target for many years in order to avoid the effects of damage caused by stress. To this end, much interest has been paid for expressing in plastids proteins, such as superoxide dismutase (*MnSOD* gene), glutathione reductase (*gor* gene) [133], dehydroascorbate reductase (*DHAR* gene), glutathione-S-transferase (*GST* gene) [134,135], and arabitol dehydrogenase (*ArDH*) [136], which all show a direct impact on the plant's tolerance to different types of abiotic stress, such as UV-B radiation, heavy metal, salt, cold, and osmotolerance [137]. Plastid expression has been used to express betaine aldehyde dehydrogenase (*badh* gene) [39], *otsB-A* operon (trehalose phosphate synthase/phosphatase) [138], homogentisate phytyltransferase (*HPT* gene), tocopherol cyclase (*TCY* gene), γ-tocopherol methyltransferase (*TMT* gene) from *Synechocystis* sp. [139], and flavodoxin (*fld* gene) from *Anabaena* sp. [140], conferring tolerance to cold stress, salinity, and drought.

The enhancement of plant tolerance against abiotic stress has been proposed, mostly targeting the glycine betaine (GB) to the chloroplast in order to increase the antioxidant pathway [134]. In this sense, choline monooxygenase (BvCMO), which catalyzes choline conversion to betaine aldehyde, has been tested in tobacco plants. Choline oxidase (*codA*) from *Arthrobacter globiformis* has been tested in potato plants to increase GB [141,142]. The results have shown plants with salt tolerance, higher relative water content levels, and normal chlorophyll content under drought stress conditions, indicating that it is feasible to improve the GB pathway in plastids.

In the search for plants that are tolerant against biotic and abiotic stresses, the chloroplast's ability to express multiple genes has also been used. Sporamin (from *Ipomoea batatas*), cystatin (from *Colocasia esculenta*), and chitinase (from *Paecilomyces javanicus*) genes were simultaneously expressed protecting against methyl viologen, salt, and osmotic stress, respectively, also displaying toxicity to *Spodoptera litura*, *Spodoptera exigua, Alternaria alternata*, and *Pectobacterium carotovorum* subsp. *carotovorum* [143].

The available reports suggest that the expression of proteins in plastids is a valuable and viable tool to improve tolerance against abiotic stress in plants. This is an issue that requires further review to establish the metabolite or the most feasible metabolic pathways that can be modified in order to produce an efficient biotic and abiotic stress tolerance, as well as the consequences on plant physiology.

5.3. Herbicide resistance

Herbicides used to eliminate unwanted plant species are an essential part of the agricultural market—driving the agronomic interest in species resistant to herbicides, another major plant biotechnology objective.

One of the most widespread chemical products to control weeds is glyphosate. This broad-spectrum post-emergent systemic herbicide inhibits EPSPS, which is required for the biosynthesis of aromatic amino acids. Glyphosate-resistant crops have been developed by modern agriculture to facilitate weed control [144]; in this regard, work has been done to incorporate new resistance to plants against other herbicides to benefit agriculture.

Although studies of herbicide-resistant plants have been carried out mainly by nuclear expression, the study of the chloroplast's capacity to express genes that can promote herbicides' resistance has already been studied (Table 3). It has been 22 years since Daniell et al. [145] reported the obtaining of glyphosate-resistant transplastomic tobacco plants by the overexpression of the wild-type petunia *EPSPS* gene that resisted ~ten times the lethal dose; since then, increased resistance has been desired through the use of synthetic EPSPS, EPSPS from other organisms such as the *CP4* gene from *Agrobacterium*, one of the most highly glyphosate-resistant EPSPS, and also by the expression of the *aroA* gene, which is indispensable for the accumulation of EPSPS [58,146,147].

On the other hand, multiple genes have been successfully expressed in plastids to confer herbicide resistance. The bacterial *bar* gene was expressed in tobacco to protect against phosphinothricin (PPT), resulting in a resistance of up to 50 mg L^{-1} [148]. Moreover, the *hppd* gene from *Pseudomonas fluorescens* was expressed in tobacco and soybean plastids, providing strong tolerance to isoxaflutole, accumulating 5% of TSP [149]. Also, sporamin, cystatin, chitinase [143], dehydroascorbate reductase (*DHAR* gene), glutathione-S-transferase (*GST* gene), and glutathione reductase (*gor* gene) [134] were expressed in tobacco chloroplast protecting against methyl viologen (Paraquat).

In addition, protection against chlorophenylthio-triethylamine (CPTA) has been obtained in tobacco and tomato plants by the expression of the bacterial lycopene β-cyclase (*crtY* gene) in plastids [73]. The protection against sulcotrione was obtained with the expression of hydroxyphenylpyruvate dioxygenase (*hpd* gene) [150], whereas protection against pyrimidinylcarboxylate, imidazolinone, and sulfonylurea herbicides was obtained via the expression of a mutated acetolactate synthase (m*ALS*) gene [151]. Recently, Stavridou et al. [135] confirmed the use of glutathione transferase (*GST* gene) in chloroplast to obtain Diquat-herbicide tolerance.

Although there have been advances in developing herbicide-resistant plants by plastid transformation, the majority of reports are on tobacco—reports on agriculturally relevant plants are limited [149]. Some aspects need to be further investigated to ensure efficient herbicide resistance through plastid transformation, such as the gene that must be expressed to provide efficient protection, the maximum expression cassette size that the chloroplast can support, the effect of these recombinant proteins on the integrity of plants and, moreover, how the protein production in floral and vegetative meristems, whose cells have fewer plastids, can be increased.

6. Chloroplast genetic engineering in monocots

Plants are an attractive system for recombinant proteins of agricultural interest, with minimal risk of contamination with animal pathogens [152]. The nuclear transformation has led to agronomically important crops' development with protection against

Table 3
Plastid transformation, advances in agronomic traits.

Gene	Product	Selection rounds	Promoter	5′UTR	3′UTR	Chloroplast region	Selection marker	Total soluble protein (TSP)	Host	Ref.
Resistance to pest and diseases										
cry2Aa2	Cry2Aa2	2	*rrn*	*	*psbA*	*trnI-trnA*	*aadA*	46.1%	Tobacco	[109109]
cry1Ia5	Cry1Ia5	4	*psbA*	*psbA*	*psbA*	*rbcL-accD*	*aadA*	3%	Tobacco	[202]
cry1Ab	Cry1Ab	3	*rrn*	*rrn*	*psbA*	*rrn16-trnI*	*aadA*	11.1%	Cabbage	[108]
cry1C	Cry1C	*	*rbcL*	*	*E. coli thra*	*rbcL-accD*	*aadA*	1%	Tobacco	[74]
cry1Ab	Cry1Ab	*	*rrn*	*T7g10*	*rbcL*	*rps12/7-trnV*	*aadA*	*a*	Soybean	[111]
cpo	Chloroperoxidase	3	*rrn*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	*a*	Tobacco	[120]
pta	*Pinellia ternata* agglutinin	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	~9.2%	Tobacco	[119]
MSI-99	Magainin-type antimicrobial peptide	3	*rrn*	*T7g10*	*rps16*	*trnI-trnA*	*aadA*	89.75 μg/g·FW	Tobacco	[118]
cry1C	Cry1C	3	*rrn*	*T7g10*	*rrnB*	*trnfM-trnG*	*aadA*	20.7 μg/g·FW	Poplar	[112]
Abiotic stress tolerance										
Gor MnSOD	*Escherichia coli* glutathione reductase Superoxide dismutase	3	*rrn*	*rbcL*	*rbcL*	*rps12/7-trnV*	*aadA*	*a*	Tobacco	[133]
DHAR GST gor	Dehydroascorbate reductase Glutathione-S-transferase Glutathione reductase	3	*rrn*	*	*psbA*	*rbcL-accD*	*aadA*	0.79%	Tobacco	[134]
fld	Flavodoxin	3	*rrn*	*psbA*	*rps16*	*rrn16-trnI*	*aadA*	~11 μmol Fld m^{-2}	Tobacco	[140]
HPT TCY TMT	Homogentisate phytyltransfera Tocopherol cyclase γ-tocopherol methyltransferase	2	*rrn*	*T7g10*	*rbcL* *rps16*	*psbH-petB* *rps2-atpI*	*aadA*	*a*	Tobacco Tomato	[139]
ArDH	Arabitol dehydrogenase	~2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	*a*	Tobacco	[136]
CMO	Choline monooxygenase	~4	*rrn*	*T7g10*	*rps*	*trnfM-trnG*	*aadA*	*a*	Tobacco	[141]
codA	Choline oxidase	*	*rrn*	*T7g10*	*rrnB*	*trnfM-trnG*	*aadA*	*a*	Potato	[142]
*	Sporamin Cystatin Chitinase	4	*rrn*	*	*psbA*	*trnI-trnA*	*aadA*	~1%	Tobacco	[143]
Herbicide resistance										
CP4	CP4 EPSPS	~3	*rrn*	*rbcL* *T7g10*	*rps*	*trnV-rps7/3*	*aadA*	~0.002% 0.3%	Tobacco	[58]
hppd	4-hydroxyphenylpyruvate dioxygenase	2	*psbA*	*psbA*	*rbcL*	*rbcL-accD*	*aadA*	5%	Tobacco Soybean	[149]
aroA	5-enoylpyruvyl shikimate-3-phosphate synthase	3	*rrn*	*	*rbcL*	*	*aadA*	*a*	Tobacco	[146]
mALS	Mutated acetolactate synthase	*	*psbA*	*	*psbA*	*rbcL-accD*	*aadA*	*a*	Tobacco	[151]
crtY	Bacterial lycopene β-cyclase	4	*atpI*	*	*rps16*	*trnfM-trnG*	*aadA*	*a*	Tobacco Tomato	[73]
hpd	4-hydroxyphenylpyruvate dioxygenase	*	*rrn*	*	*	*rps12-orf131*	*aadA*	*a*	Tobacco	[150]
bar	Phosphinothricin	3	*rrn*	*	*psbA*	*trnI-trnA*	*aadA*	*a*	Tobacco	[148]

a Indicates observed adequate gene expression, but no quantification.
* Indicates not reported.
FW: Fresh weight

insects, herbicide resistance, drought tolerance, salt tolerance, and phytoremediation. Although there have been advances in developing plants through plastid transformation, monocot crops limit this technology's expansion [5]. Though chloroplast genetic transformation is still very incipient in monocots and there are limited reports, these begin to show strategies that must be considered to address this field, which has the potential to change agrobiotechnology.

The first attempts date from 1991 when Daniell et al. [153], using a double *psbA* promoter from pea, achieved the transitory expression of *uidA* gene within the chloroplasts of wheat although without stable expression; however, their results showed that dicot chloroplast promoters could be functional in the chloroplasts of monocots. Eight years later, using callus, Khan and Maliga [154] reported a stable plastid transformation in rice (*Oryza sativa* cv. Taipei 309), introducing the biofunctional marker gene FLARE-S (Fluorescent Antibiotic Resistance Enzyme, Spectinomycin, and Streptomycin) into *rrn16-rps12|7* chloroplast regions, obtaining heteroplasmic plants; however, the transgene transmission to subsequent generations could not be verified. Later, using calli, the *aadA* and *sgfp* genes were inserted into *trnI-trnA* region of the chloroplast genome from japonica rice (*Oryza sativa* L. cv. Hwa-Chung) with lower transformation efficiency and remaining heteroplasmy, but producing fertile plants with transgenes expressed in the progeny [155]. These results on monocots' plastids are significant because cereals are an essential source of calories and protein in the human diet [156].

In recent years, *gfp* and *nptII* genes were inserted into *atpB-rbcL* intergenic region from wheat (*Triticum aestivum* L. cv. Bobwhite), obtaining homoplasmic plants in T_0 generation using scutella, and heteroplasmic plants with immature inflorescences [157]. Although the results were promising, they could not be confirmed and the results were retracted, pending further experimentation [158].

Currently, satisfactory progress has been made in rice. In 2018, callus from the japonica rice line 19 was transformed with the

soluble-modified Green Fluorescent Protein (*smGFP* gene) and the selection marker gene hygromycin phosphotransferase (*hpt* gene) by inserting them into the *trnI-trnA* region of the rice chloroplast genome, obtaining homoplasmic plants after one year of screening [5]. Despite this success, the material did not reach the seed stage, so it was not possible to determine whether the plants were sterile or not. Hence, the chloroplast transformation in monocots is still a proof of concept.

Zea mays is an efficient platform to study plant genes' functionality, but above all, because it is one of the most important crops worldwide. Unfortunately, maize plastid transformation has never been successful.

Recently, our research group constructed two vectors to transform maize (*Zea mays* tropical variety "LPC13" line) and wild type grass *Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Steud, respectively, which contain the Green Fluorescent Protein reporter (*mgfp5* gene) and the selection marker gene hygromycin phosphotransferase (*hpt* gene), driven by an *rrn16* promoter flanked by the *trnA-trnI* chloroplast regions [159]. Both vectors were tested using immature embryos from young maize seeds observing green fluorescent points in scutellum, suggesting that vectors' functionality can direct genes to plastid monocots, but this is not yet confirmed.

Monocots have been recalcitrant to plastid transformation due to several hurdles that need to be resolved promptly, such as the gene transfer methods, the development of an efficient plant regeneration media, and the strategies to reduce DNA loss content per plastid in seedlings on light conditions. It could decrease the number of transformed chloroplast, preventing regenerate plants [160]. In this sense, a significant limitation is the low transformation efficiency in monocots; however, the use of Transcription Activator-Like Effector Nucleases (TALENs) has given promising results [161].

Another limitation is the gene expression in non-green plastids, as the transformation of cereals starts from non-green tissues like scutella or callus. Most of the genes in non-green plastids are highly down-regulated, except for *accD* and *clpP* genes [162], which are important for the lipid metabolism and proteolytic machinery in the plastid. Hence, the use of regulatory sequences from these genes to express genes in the plastids of cereal crops may allow the recovery of transformants in selective media.

A limitation of chloroplast transformation is the necessary development of a new vector for each plant species. Although universal vectors containing the *trnI-trnA* genes from the inverted repeat region have been proposed [145] (as well as vectors containing the *trnfM-trnG*, *ndhB-trnL,* and *rrn16-trnV* regions [163]), the intergenic spacer regions in chloroplast genomes are not well-conserved [4]. The loss of homology between sequences from transformation vectors and chloroplast genome decreases the frequencies of transformation [37,164,165]. It could also decrease the expression level up to 90% [4,81,166]; therefore, currently, there are no vectors used in all crops.

It has been observed that complete chloroplast genomes can move between cells from one species into another across the graft junction as an organelle capture process. This raises the possibility of grafting donor plant tissue containing transformed plastids into a recipient plant tissue, allowing the migration of transplastomic plastids into the untransformed tissues [167,168]. Perhaps this strategy could be used in monocots—this remains unexplored.

Despite the recalcitrance of monocots to plastid transformation, it is evident that plastid technology is a tool that can enhance the biosafety of genetically modified plants. Efforts must be focused on solving the limitations in cereal crops because they will extend plastid transformation to a broader set of plant species.

7. Pleiotropic effects in transplastomic plants

In most reports, the expression of recombinant proteins in plastids does not alter the plant's phenotype. Some reports indicate that the expression of recombinant proteins could generate abnormal phenotypes that include yellow leaves, reduced growth of transformed plants, and male sterility. In many cases, the abnormal phenotype gradually disappears or does not affect the growth and development of plants [23,169]; however, in others, this phenotype affects plants' development [6] (Table 4).

Scotti et al. [170] reported a slower growing and chlorotic phenotype from *in vitro* culture to maturity and a protein decrease of up to 50% when the HIV-1 $Pr55^{gag}$ polyprotein was expressed in tobacco chloroplasts. Similar phenotypes were obtained with the expression of HIV-1 $protein^{Nef}$ in tobacco and tomato plants; 30% of transplastomic lines had yellow tissues [171]. Rigano et al. [172] expressed the A27L immunogenic protein of the vaccinia virus. Although neither flowering nor seed setting was affected in transplastomic lines, the plants showed slower growth and had a slightly chlorotic phenotype. Waheed et al. [173,174] used the *L1_2xCysM* gene expression obtaining a pale microcalli and male-sterile transplastomic tobacco lines, with flowers that fell before maturity or did not produce seeds, and that could only produce seeds by cross-fertilization with pollen from wild-type tobacco. Further, the expression of aprotinin showed plants with a pale green phenotype that also grew more slowly compared to wild-type tobacco and with delayed seed production [175], the same as E7 Human papillomavirus antigen [86], and Human epidermal growth factor (*hEGF*) [7].

Sometimes, abnormal phenotypes have some utility and can be used as biosafety improvement or adaptation in modified organisms. For example, Ruiz and Daniell [176] used plastid transformation via an abnormal phenotype product of the β-ketothiolase expression in plastid-obtained plants with the accelerated development of anthers, which caused a collapsed morphology of the pollen grains. This affected their maturation and led to sterility. Through this, they designed a cytoplasmic male sterility system in plants. An abnormal phenotype was obtained by Jin et al. [177] with the expression of a β-glucosidase in tobacco chloroplast, which showed an increase in biomass, height, leaf area, and trichome density due to an increase in gibberellins that produced sugar esters in transplastomic exudates, which also decreased the populations of whitefly and aphid, resulting in a new strategy to design tall plants with biopesticides.

To date, it is not well understood what causes all these effects or what the solution is. However, Ruiz and Daniell [176] proposed that abnormal phenotype is related to the type of gene expressed. In this respect, the fact that there are reports with protein expression with less than 2% of TSP with pleiotropic effects [173] and reports with an overexpression of up to 72% of TSP without adverse effects on plants [4] suggests that pleiotropic effects are not a consequence of the overexpression of foreign proteins. Perhaps detrimental effects can be attributed to various factors, such as the interference of the recombinant proteins with thylakoid structure and function, interference of novel open reading frames within the cytoplasmic metabolism, or lower levels of ATP production [178].

Whatever the reason for pleiotropic effects, detailed studies regarding the metabolic pathways affected within chloroplasts (or the interaction of the recombinant proteins with the chloroplast's photosynthetic machinery) are necessary to lessen the adverse effects. The use of strategies for inducible expression in the chloroplast transformation can circumvent the observed detrimental effects in plants and about this, significant progress has been made in recent years [179].

Table 4
Plastid transformation reports that have shown pleiotropic effects.

Gene	Product	Selection rounds	Promoter	5′UTR	3′UTR	Chloroplast region	Selection marker	Total soluble protein (TSP)	Host	Pleiotropic effect	Ref.
CelA-CelB	Dicistronic cellulases type A-B	3	*rrn*	*rbcL*	*rbcL*	*rrn16-3′rps12*	*aadA*	*a*	Tobacco	Variegate phenotype	[23]
CrtW	*β*-carotene ketolase	*	*rrn*	*rrn*	*rps16*	*rbcL-accD*	*aadA*	*a*	Tobacco	Slow growth	[169]
CrtZ	*β*-carotene hydroxylase										
gag	$Pr55^{gag}$	2	*rrn*	*rbcL*	*rbcL*	*rps12/7-trnV*	*aadA*	6.75%	Tobacco	Chlorotic phenotype, slow growth, and decreased protein	[170]
p24-Nef	HIV antigens p24-Nef	4	*rrn*	*T7g10*	*rrnB*	*trnfM-trnG*	*aadA*	40% 2.5%	Tobacco Tomato	Chlorotic phenotype	[171]
A27L	A27L immunogenic protein	2	*rrn*	*T7g10*	*rbcL*	*rps12/7-trnV*	*aadA*	18%	Tobacco	Slow growth and slightly chlorotic phenotype	[172]
E7	E7 Human papillomavirus antigen	3	*psbA*	*psbA*	*rps*	*rrn16-trnI*	*aadA*	~8%	Tobacco	Sterile lines	[86]
L1_2xCysM-LTB	HPV-16 L1-LTB	5	*rrn*	*	*rbcL*	*trnN-trnR*	*aadA*	2%	Tobacco	Pale microcalli and no seed production	[173]
L1_2xCysM	HPV-16 L1	6	*rrn*	*T7g10*	*rbcL*	*trnN-trnR*	*aadA*	1.5%	Tobacco	Male sterility	[174]
BPTI	Aprotinin	1	*psbA*	*psbA*	*rbcL*	*rbcL-accD*	*aadA*	~0.5%	Tobacco	Pale green phenotype and slow growth	[175]
Pha	β-ketothiolase	2	*psbA*	*psb*	*psb*	*trnI-trnA*	*aadA*	*a*	Tobacco	Male sterility	[176]
hEGF	Human epidermal growth factor	3	*psbA*	*psbA*	*rps*	*rrn16-trnI*	*aadA*	*a*	Tobacco	Slow growth	[7]
SAG1	SAG1 surface antigen	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	2.5%	Tobacco	Slow growth and chlorotic phenotype	[201]
GAA	Alpha glucosidase	2	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	0.21%	Tobacco	Chlorotic phenotype	[99]
MSI-99	Magainin-type antimicrobial peptide	3	*rrn*	*T7g10*	*rps16*	*trnI-trnA*	*aadA*	89.75 μg/g·FW	Tobacco	Slow growth	[118]
bgl1C	*β*-glucosidase	3	*rrn*	*T7g10*	*rbcL*	*trnfM-trnG*	*aadA*	40%	Tobacco	Chlorotic phenotype	[203]
cel6B	Exocellulase										
cel9A	Endocellulase										
xeg74	Xyloglucanase										
bgl1	*β*-glucosidase	3	*psbA*	*psbA*	*psbA*	*trnI-trnA*	*aadA*	*a*	Tobacco	Increase in biomass, height, foliar area and trichome density	[177]

a Indicates observed adequate gene expression, but no quantification.
* Indicates not reported.
FW: Fresh weight.

8. Conclusions and future remarks

The growing demand for food for an expanding population forces the development of modified crops with high-quality by-products. Chloroplast genetic engineering offers a tremendous opportunity to express a wide variety of proteins with industrial value, which, although it is still in development, has proven its potential in agricultural crops and biopharmaceutical elements. Even though chloroplast transformation has been achieved in several crops, the most crucial task for the coming years will be to implement plastid transformation in essential crops, such as cereals. The combination of effective tissue culture systems, gene expression in non-green plastids, optimization of the regulatory sequences, and transformation methods can help overcome limitations, such as low transformation efficiency, difficulty in obtaining homoplasmic plants, and would allow extending plastid transformation to a broader set of plant species, including recalcitrant crops.

The future of chloroplast engineering still has some obstacles to overcome. Despite these obstacles, it can reduce production costs, produce highly bioavailable compounds, and eliminate the environmental impact problems currently associated with nuclear genetically modified crops.

Conflict of interest

The authors declare that they have no conflict of interest.

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