

## *Retraction*

# **Retracted: Stable Plastid Transformation for High-Level Recombinant Protein Expression: Promises and Challenges**

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This paper [1] has been retracted as it is found to contain a substantial amount of material from the paper “Chlamydomonas Reinhardtii Chloroplasts as Protein Factories” by Stephen P. Mayfield, Andrea L. Manuell, Stephen Chen, Joann Wu, Miller Tran, David Siefker, Machiko Muto, and Julia Marin-Navarro published in *Current Opinion in Biotechnology* in April 2007.

### **References**

- [1] M. Gao, Y. Li, X. Xue, X. Wang, and J. Long, “Stable plastid transformation for high-level recombinant protein expression: promises and challenges,” *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 158232, 16 pages, 2012.

## Review Article

# Stable Plastid Transformation for High-Level Recombinant Protein Expression: Promises and Challenges

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Plants are a promising expression system for the production of recombinant proteins. However, low protein productivity remains a major obstacle that limits extensive commercialization of whole plant and plant cell bioproduction platform. Plastid genetic engineering offers several advantages, including high levels of transgenic expression, transgenic containment via maternal inheritance, and multigene expression in a single transformation event. In recent years, the development of optimized expression strategies has given a huge boost to the exploitation of plastids in molecular farming. The driving forces behind the high expression level of plastid bioreactors include codon optimization, promoters and UTRs, genotypic modifications, endogenous enhancer and regulatory elements, posttranslational modification, and proteolysis. Exciting progress of the high expression level has been made with the plastid-based production of two particularly important classes of pharmaceuticals: vaccine antigens, therapeutic proteins, and antibiotics and enzymes. Approaches to overcome and solve the associated challenges of this culture system that include low transformation frequencies, the formation of inclusion bodies, and purification of recombinant proteins will also be discussed.

## 1. Introduction

The demand for recombinant proteins such as biopharmaceutical proteins and industrial enzymes is expected to rise dramatically in the near future. However, the current capacity and cost of production for most recombinant proteins limits their availability [1]. Therefore, the strong global demand for low-cost and high-yield recombinant proteins is the impetus driving molecular farming, particularly in developing nations [2]. Commercial production of such recombinant proteins has traditionally relied on bacterial fermentation or mammalian cell-based production. However, limitations including cost, scalability, safety, and

protein authenticity with these expression systems have prompted research into alternative platforms [3, 4].

Recently, plant-based systems potentially provide a low-cost alternative for the production of recombinant proteins. Strategies for plant transformation contain stable nuclear transformation, stable plastid transformation, plant cell-suspension, and transient expression systems [5]. Plant cell suspension cultures have several advantages including the capacity for shorter life cycles, independence from environmental effects such as climate, soil quality, season, day length and weather, the lack of biosafety issues such as gene flow via pollen, and the possibility of bacterial contamination from the plant growth environment [6]. But, the yield

and quality of recombinant proteins in plant cell culture-based expression systems need to be further improved. In addition, the transient expression systems, which are perhaps the fastest and the most convenient production platform for plant molecular farming, are mainly used for quick validation of expression constructs [7].

Production of recombinant proteins in transgenic plants was initially based on integration of a target gene into the nuclear genome and later included transformation of the chloroplast genome [1]. Stable nuclear transformation leads to the expression of the transgene after integration with the host genome. This transformation confers stably inheritable traits that were not present in the untransformed host plant [5]. Plant-based systems combine advantages of both production systems: as higher eukaryotes, plants synthesis complex multimeric proteins with posttranslational modifications closely resembling mammalian modifications. In addition, production in plants eliminates the risk of product contamination by human pathogens possibly hidden in mammalian cell lines or in their complex organic production media [8]. However, except for few recombinant proteins, most often very low expression levels of foreign proteins (less than 1% of the total soluble protein, TSP) were observed in nuclear transgenic plants. Also, gene silencing can occur in nuclear transformation, which results in lower expression of recombinant proteins [9]. The impinging problems of nuclear transformation associated with position effects due to random gene integration, and safety due to environmental dissemination of genes by pollen has hampered its expediency for commercialization [10]. For commercial exploitation of the therapeutic proteins and vaccine antigens, high and reliable levels of expression are required, which could be achieved by alternative approaches [9].

Plastid transformation provides a valuable alternative to nuclear transformation because it combines numerous advantages, especially high expression levels that the nuclear transformation lacks. This review focuses on stable plastid transformation in plant. Here in, we give main advantages on plastid information, factors for high-yield production, the expression level of recombinant proteins in plastid, the challenges directions in the development and commercialization of recombinant proteins in plastid expression system are discussed.

## 2. Advantages of Plastid Expression Systems

Plant cells contain three genomes: a large one in the nucleus and two smaller ones in the mitochondria and plastids. Plastids are a group of organelles that include the sites of photosynthesis of chloroplasts, as well as several other differentiation forms, including the carotenoid-accumulating chromoplasts in flowers and fruits, and the starch-storing amyloplasts in roots and tubers. As semiautonomous organelles, each cell contains a large number of plastids, ~100 chloroplasts per cell and each chloroplast contains about 100 genomes. Therefore, plastid transformation permits the introduction of thousands of copies of transgenes

per plant cell. It dramatically enhances the protein production in the cell [11, 12]. Though both plastid transformation and nuclear transformation are stable recombinant protein expression systems in plants, the protein expression level is far higher in the former transformation than that of the latter transformation.

The issue of transgene containment and prevention of its escape into the environment and into wild-type plant populations is becoming increasingly relevant due to the exponential growth of the use of genetically modified plants in agriculture [13, 14]. Generally, nuclear transgenes can be transmitted by pollen and thus require additional genetic modifications to ensure transgene containment, such as engineering of male sterility [15]. However, chloroplast genomes defy the laws of Mendelian inheritance in that they are maternally inherited in most species and the pollen does not contain chloroplasts. The chloroplast expression system has a natural biocontainment of transgene flow by outcrossing. In this regard, transplastomic plants are much safer than plants with nuclear transgenes. Therefore, the plastid expression system is an environmentally friendly approach and is allaying public concerns [5, 9].

In addition, transgene integration into the plastome is based on two homologous recombination events between the targeting regions of the transformation vector and the wild-type ptDNA (plastid genome or plastome) [16]. Chloroplast transformation eliminates the concerns of position effect, frequently observed in nuclear transgenic lines [17]. Recently, it has been demonstrated that the extent of similarity between the plastidial sequences involved in homologous recombination is important to ensure high transformation efficiency [18, 19]. Hence, the lack of transgene silencing has been observed in chloroplast transformation accompanied with higher expression levels than in nuclear transgenic plants. For example, no gene silencing has been observed in spite of high translation levels, up to 46.1% TSP. It has been observed that there is also no gene silencing when transcripts accumulated 169-fold and 150-fold higher in transgenic chloroplasts than nuclear transgenics [20, 21].

Besides previous advantages, it is possible to express several genes using a single promoter which makes the plastid transformation approach a highly attractive method [22]. Several heterologous operons have been expressed in transgenic chloroplasts, and polycistrons are translated without processing into monocistrons [23]. Factually, most plastid genes are arranged in operons, which are transcribed as polycistronic mRNAs. The processing mechanisms for translation regulation in chloroplast genes mainly include posttranscriptional RNA processing and intercistronic processing. Posttranscriptional RNA processing of primary transcripts represents an important control which relies more on RNA stability than on transcriptional regulation of chloroplast gene expression. RNA stability is mainly influenced by the presence of 5'-UTRs, nucleus-encoded factors and 3'-UTRs. Intercistronic processing (i.e., RNA cutting) refers to many primary polycistronic transcripts in plastids undergoing posttranscriptional cleavage into monocistronic or oligocistronic units. This process enhances translation of chloroplast operons such as *psbB*, *petD*, and *pet* clusters

in maize. Additionally, different species may experience various processing mechanisms for the same gene cluster. On the other hand, some polycistronic mRNAs in plastids are translatable and do not undergo posttranscriptional cleavage into monocistronic units. These polycistronic mRNAs often have canonical SD sequences upstream of each individual cistron. Simultaneously, some polycistronic transcripts are not translatable that endonucleolytic processing can be a prerequisite for protein biosynthesis [23, 24]. Chaperones, which present in chloroplasts, facilitate chloroplasts to show the correct folding and assembling complex mammalian proteins [25]. This was demonstrated through the formation of functional protein such as interferon alpha and gamma as well as cholera toxin-B subunit (CTB) in transgenic chloroplasts [9]. Further, chloroplasts can also be a good place to store the biosynthetic products that could otherwise be harmful when accumulated in cytosol [26]. As described above, CTB was toxic when expressed and accumulated in the cytosol in very small quantities. However, CTB was accumulated in large quantities and it had no toxic effect in chloroplast [27]. Trehalose is used as a preservative in the pharmaceutical industry. Similarly, trehalose was toxic when accumulated in cytosol but was nontoxic when compartmentalized within chloroplasts [20].

### 3. Factors for High-Yield Production in Chloroplast Expression Systems

Chloroplast offers an alternative stable expression system to nuclear transformation. Highly polyploid nature of the plastid genome will allow the integration of thousands of copies of transgenes per cell. This will result production of very high levels of proteins in the transgenic plants produced by plastid transformation [22]. The regulation of recombinant protein expression is a complex system. It includes the interacting elements and the extent of interdependence between different factors, which is not completely understood. Some factors, such as promoters, UTR sequences, codon optimization, post-translational modification, construction of transcriptional fusions, protease activity, as well as accumulation of toxic recombinant protein in chloroplast have been concentrated. Recent progress and development on these factors affecting recombinant protein levels are reviewed here.

**3.1. Codon Optimization.** The genetic code is made up of many redundant triplets that encode for the same amino acid. This implies many alternative nucleic acid sequences can encode a protein. Since the rules for deciphering a DNA sequence to determine the amino acid sequence of the encoded protein were established over 40 years ago, the genomes of different organisms, and the different genomes of single organisms, employ codon biases as mechanisms for optimizing and regulating protein expression are well established [28]. Optimizing the codon usage of most heterologous genes further reflects that codon biases can

increase their expression efficiency by increasing their translation rates, and may decrease their susceptibility to gene silencing [29].

The expression level of recombinant protein was very low when recombinant genes were directly taken from other systems and were not optimized for expression in *Chlamydomonas reinhardtii* chloroplasts [30]. As described above, codon bias is the most important determinant of protein expression in prokaryotic genomes [31], and adjustment of codons in transgenes is necessary for high level (i.e., commercially viable) expression [32]. This was further evidenced by a green fluorescent protein (gfp) reporter gene in accordance with such codon bias. The codon-optimized gfp and nonoptimized native gfp genes were transformed into chloroplasts, both driven by the same promoter and UTR [33]. The codon-optimized gfp gene resulted in an 80-fold increase in GFP accumulation compared with the nonoptimized version. Transformation of a codon-optimized human antibody gene [34] and codon-optimized luciferase gene [32] in *C. reinhardtii* chloroplasts also confirmed that codon bias plays a significant role in protein accumulation. Thus, increased protein production in these strains highlights the necessity for codon optimization of any gene for which high levels of protein production are desired. Further, in a recent study, a hepatitis C virus core polypeptide expressed in chloroplasts, the results suggested that the codon-optimized gene increased monocistronic core mRNA levels by at least 2-fold and core polypeptides by over 5-fold, relative to the native viral gene [35].

Recently, the codon adaptation index (CAI) is used as a quantitative tool to predict heterologous gene expression levels based on their codon usage. As the chloroplast, mitochondrial, and nuclear genomes may exhibit different codon biases, genome-specific CAI values should be used for optimal translational [36]. Codon optimization is an effective and necessary step in gene sequence optimization, and one relatively simple to address with recent advances in DNA synthesis technology. Analysis of codon usage in chloroplast genes by e-CAI evaluation software (<http://genomes.urv.es/CAIcal/E-CAI/>) showed that translational selection does indeed operate for a majority of genes in the chloroplast. However, codon optimization is not the only factor to be considered for selection a desired gene for high level expression of recombinant protein in plastid transformation. Further, the work in [37] describes an "optimal" gene which the codon choices do not limit expression is a desired need in the future study.

**3.2. Promoters and UTRs.** The gene expression level in plastids is predominately determined by promoter and 5'-UTR elements. Promoters contain the sequences which are required for RNA polymerase binding to start transcription and regulation of transcription. As the strength and expression profile of the key regulatory element "promoter", it plays an important role in driving the transcription to achieve high level of transcription. Hence, in order to obtain high-level protein accumulation from expression of the transgene, the first requirement is a strong promoter to ensure high levels

of mRNA. Chloroplast-specific promoters have also been utilized for targeting the foreign protein expression into chloroplasts [12]. For example, the 16S ribosomal RNA promoter (Pr<sub>rrn</sub>) like *psbA* and *atpA* gene promoters are commonly used for chloroplast transformation. Several molecules including CTB, LTB, protective antigen, insulin, or vaccines have been produced in chloroplasts using either Pr<sub>rrn</sub> or *psbA* promoter [27, 31, 38–41]. These promoters drive the high level of recombinant protein expression in plastid transformation. Other promoters may be found from Dow AgroSciences LLC which has secured exclusive rights to Chloroplast Transformation Technology (CTT) from Chlorogen, Inc. (<http://www.dowagro.com/search.aspx?q=promoter>).

Stability of the transgenic mRNA is ensured by the 5'-UTR and 3'-UTR sequences flanking the transgenes. The 5'-UTR is very important for translation initiation and plays a critical role in determining the translational efficiency. This was also evidenced by a series of studies in plastid transformation. For example, transcriptional efficiency was shown to be regulated by both chloroplast gene promoters as well as sequences contained within the 5'-UTR [42]. A variety of studies have revealed that translational efficiency is a rate limiting step for chloroplast gene expression [43] and have shown that the 5'-UTRs of plastid mRNAs contain key elements for translational regulation [44]. Additionally, sequences found within the 5'-UTR are also important for optimal levels of transcription, although the nature of these internal enhancer sequences has yet to be defined [42]. The 3'-UTR plays an important role in gene expression as it contains message for transcript polyadenylation that directly affects mRNA stability [45]. The most commonly used 5'-UTR and 3'-UTR are *psbA*/*TpsbA* [46–50]. However, in a recent study, a *gfp* reporter gene and the 5'- and 3'-UTRs from five different chloroplast genes were used to construct a series of chimeric genes in the chloroplast genome. The results showed that the highest levels of recombinant protein expression were obtained using either the *atpA* or *psbD* 5'-UTRs, while the nature of the 3'-UTR invariably had little effect on reporter protein accumulation [51]. Hence, more 5'- and 3'-UTRs and the sequences within the coding region on the expression of recombinant protein in plastid should be further studied.

**3.3. Genotypic Modifications.** A synthesized gene is generally modified from the natural version because natural genes are often poorly expressed in heterologous hosts, even when the expression system is related to the organism from which the gene originated [37]. Transgenes are inserted in the chloroplast genome by homologous recombination, which implies that each transformant obtained should be identical if using a single integration vector. Identical recombinant protein expression profiles for each transformant are therefore expected [36]. However, Surzycki et al. [31] reported the protein yields varying from 0.88% to 20.9% total cell protein (TCP) in transgenic lines obtained from a single biolistic transformation in chloroplast. This variation may be due to genotypic modifications resulting from the transformation process. The low expression levels of transgenic proteins may

depend more on these modifications than on the selection of promoters, UTRs, or insertion sites.

**3.4. Endogenous Enhancer and Regulatory Elements.** As previously reported, for posttranscriptional regulation in chloroplast, the light plays a vital role in the translation of many chloroplast mRNAs [43, 52]. The highest level of light induction is for the *psbA* mRNA which encodes D1, a core protein of photosystem II [53]. Additionally, the *psbA* 5'-UTR is capable of conferring light-regulated translation to recombinant mRNAs [34, 51]. This offers the potential to regulate recombinant protein expression, which might be necessary to express proteins that are not well tolerated by the chloroplast. Further, *psbA*-driven heterologous protein expression is increased when the endogenous *psbA* gene is deleted [32]. This may be due to either the removal of autoattenuation from D1 protein feedback [54] or to reduced competition with endogenous *psbA* for limited transcription or translation factors. In fact, this is a process that we refer to as control by epistasy of synthesis (CES process) and occurs during chloroplast protein biogenesis in *C. reinhardtii* [55]. The synthesis of a CES subunit is markedly reduced in the absence of its assembly partners, which involves negative feedback or feedback inhibition [56, 57]. Therefore, in *C. reinhardtii* chloroplast studies, gene products, including cytochrome *f*, photosystem I (PSI), regulate the translation of their own mRNA through feedback inhibition [54, 57]. Nevertheless, the low expression levels of heterologous genes were observed in microalgal chloroplasts but not in tobacco chloroplast expression systems, in which this inhibition is not observed. In addition, placing the chloroplast transgene under the transcriptional regulation of an inducible factor is for inducible expression of recombinant proteins in higher plant chloroplasts [58, 59]. Further, it has been evidenced that a large set of nucleus-encoded factors act mostly at posttranscriptional steps of chloroplast gene expression. Among these proteins, the Nac2 protein of *C. reinhardtii*, is specifically required for the stable accumulation of the *psbD* mRNA encoding the D2 reaction center polypeptide of PSII [60].

Generally, an intrinsic helicase which exists in ribosomes has the ability to allow translation through even very strong hairpins and to preclude many structures from limiting the translation rate [61]. Hence, an actively translated message can be densely packed with ribosomes, unwinding structure as they move along. The rates of ribosome binding and clearing of the ribosome-binding site (RBS) after initial elongation (approx. 13–20 codons) play an important role in translational initiation. Slow translation through the initial leader may reduce or eliminate any benefits of a strong RBS sequence. Gene design strategies often seek to minimize mRNA structure. Structures that involve or otherwise occlude the RBS and/or start codon in genes expressed in prokaryotes can impair expression, presumably by interfering with ribosomal binding and translational initiation [62, 63]. An algorithm, which designs prokaryotic RBSs to achieve desired rates for initiation of translation considering the structure of the mRNA and

the affinity of the RBS for the ribosome, has been recently developed (<https://github.com/hsalis/ribosome-binding-site-calculator>).

The fusion of recombinant products to native proteins has also resulted in an increase of protein yield. It has been reported that the endogenous rubisco LSU protein was fused to a recombinant luciferase through a cleavable domain in algal chloroplasts. This resulted in a 33-fold increase in luciferase expression compared to luciferase expressed alone. The results suggest that recombinant protein accumulation can be enhanced by fusion with a native protein. Also, the liberation of recombinant proteins from the native ones would simplify product purification and increase the yield of recombinant protein [64].

**3.5. Posttranslational Modification and Proteolysis.** Chloroplasts lack the machinery to perform complex posttranslational modifications, like glycosylation, on proteins. So, proteins not requiring posttranslational modifications can be expressed in chloroplast expression systems. Many proteins do require posttranslational modifications not performed in the prokaryotic plastids. As we know, nonglycosylated antibodies have greatly reduced activation of complement and somewhat reduced Fc-mediated binding in activation of antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, complement fixation and ADCC activation are not required for antigen binding. Moreover, therapeutic antibodies that function to sequester molecules or block binding sites do not require the activation of ADCC and strive to avoid activation of complement. Therefore, chloroplast-expressed nonglycosylated antibodies might actually be superior to glycosylated antibodies for some therapeutic uses [65]. In some cases, for instance, for the production of the therapeutic protein human alpha1-anti-trypsin (A1AT) as well as *Plasmodium falciparum* surface protein 25 (Pfs25) and 28 (Pfs28), the absence of glycosylation could be considered an advantage. Human alpha1-antitrypsin (A1AT), a major therapeutic protein, is that mature A1AT is a glycosylated protein containing three N-linked carbohydrate sidechains. Though the glycosylation is important for the half-life of A1AT in the plasma, it is not required for the binding to elastase. As a consequence, the production of an active unglycosylated version in plants can be envisaged. Nadai and his colleagues have produced A1AT, in genetically engineered tobacco plastids. These chloroplast-made therapeutic proteins are fully active and bind to porcine pancreatic elastase [66]. Malaria transmission blocking vaccine candidates, *Plasmodium falciparum* surface protein 25 (Pfs25) and 28 (Pfs28), are structurally complex aglycosylated outer membrane proteins that contain four tandem epidermal growth factor-like (EGF) domains, each with several disulfide bonds. The chloroplast of green algae can fold complex proteins and make disulfide bonds, but lacks the machinery for glycosylation. Thus, these proteins have been produced in chloroplast of green algae which are structurally similar to the native proteins and antibodies raised to these recombinant proteins recognize Pfs25 and Pfs28 from *P. falciparum* [67].

Since the first evidence of disulfide-bond formation of human somatotropin has been expressed in tobacco chloroplasts [41], many recombinant proteins which contain the expected disulfide bonds have been expressed in chloroplasts of both higher plants and *C. reinhardtii* [48]. Consequently, chloroplasts could be an excellent system for the expression of proteins that require structural disulfide bonds. The chloroplast proteins responsible for disulfide-bond formation could be the same proteins used to transduce the light activation signals and used to regulate chloroplast translation, as one of these redox-dependent proteins is a protein disulfide isomerase [65]. As discussed above, Pfs25 and Pfs28 expressed in chloroplast of green algae further evidenced that chloroplast could fold complex proteins and make disulfide bonds and form functional proteins [67].

As we know, the proteases of prokaryotic origin play critical roles in chloroplast development and maintenance [68]. Clearly, except the chloroplast processing peptidases which cleave transit peptides, processive proteases such as the serine ATP-dependent Clp protease, the ATP-dependent metalloprotease FtsH and the serine-dependent DegP protease, degrade abnormal soluble and membrane-bound proteins, unassembled proteins and the D1 protein of PSII, as well as misfolded and periplasmic proteins, respectively. The identity of the cross-reacting protein of ATP-dependent Lon protease still needs to be confirmed [69, 70]. Factually, chloroplasts of both higher plants and algae contain proteases commonly found in bacteria; Clp, Deg, and FtsH proteases are all found in the nuclear genome of *C. reinhardtii* with at least one ortholog of each targeted to the chloroplast [69, 71]. However, these are a minor proportion of the proteases normally encountered in the cytoplasm of eukaryotic cells. Therefore, the chloroplast could potentially be a more sheltered environment for the production of proteins that are particularly susceptible to proteolysis [65]. The level of foreign protein accumulation results from a balance between rates of protein synthesis and degradation, the latter of which is increasingly found to impact recombinant product yields. Proteolytic enzymes, which are essential for endogenous protein processing, may lead to the degradation of foreign proteins after synthesis, or interfere with their correct assembly and posttranslational modification. Proteolysis may also lead to inconsistent results and to difficulties in downstream processing or purification due to degraded or nonfunctional protein fragments [36]. Hence, several strategies are available to minimize proteolytic degradation of foreign proteins in plants. For example, the coexpression of protease inhibitors has proven useful in increasing recombinant protein yields in plants, without affecting normal growth and development [36].

#### 4. High-Level Expression of Recombinant Proteins in Plastids

Attainment of high expression level of foreign proteins in plastids is a major breakthrough, which makes this system ideal for large-scale production of recombinant protein [9]. As previously reported, recombinant proteins expressed

from chloroplast genomes normally make up 5–25% of TSP [72, 73]. Some chloroplast transgenes have even been reported to accumulate to levels of 72% TSP [18], which is over to recombinant protein expression levels in bacteria. In contrast, the recombinant protein expression levels in nuclear do not exceed 1-2% TSP in most cases. For example, the expression level of human interferon-gamma (hINF- $\gamma$ ) in chloroplast was 100 times higher than those of hINF- $\gamma$  in nuclear [74]. In another study, a phage-derived protein antibiotic, PlyGBS, expressed from a chloroplast transgene accumulated to extremely high levels, exceeding 70% TSP [75]. Thus, if plants are intended to be used as bioreactors for large-scale production of recombinant proteins, chloroplast, rather than nuclear, genome should be targeted for genetic modification [76].

**4.1. Vaccine Antigens.** A vaccine is an antigenic preparation used to establish immunity against a disease and the main aim of the vaccination is to eradicate infectious diseases. The development and use of vaccines represent one of the greatest achievements in medical history. Today, numerous life-threatening diseases can be prevented efficiently by immunization, and one of them, smallpox, has even been eradicated [77]. The transplastomic plants have emerged as an attractive production system for vaccines which includes the often attainable high antigen yields, the low production costs once stable lines established, and the potential of producing orally applicable (as a result of high expression levels). Several vaccine candidates have been produced successfully via plastid transformation in the past few years [9]. This demonstrates that transplastomic plants, as a second-generation expression system, have great potential to fill gaps in conventional production platforms. A salient feature of plastids is that they combine characteristics of prokaryotic and eukaryotic expression systems [78]. Vaccines produced in plastid vaccines were proved to be fully functional and able to elicit the appropriate immune responses in experimental animals and to protect against toxin or pathogen challenges. Several recent reviews [16, 78–80] describe the production of vaccine antigens in transplastomic plants. Herein, we will concentrate on the relative high expression level of recombinant antigens produced in plastid.

CTB is responsible for inducing both mucosal and serum immunity. Since the cholera toxin is internalized by the receptors present on mucosal lining, the CTB was one of the early toxins selected for testing the concept of edible vaccines. Further, CTB being a bacterial protein is not glycosylated in native form. Hence, its feasibility for developing vaccine antigen has been examined by expressing the gene in plants both by transformation into chloroplastic and nuclear genome [81]. CTB subunit, the first vaccine expressed in tobacco chloroplasts, showed the expression level as high as 4.1% TSP [27]. Conversely, the tobacco leaves expressed CTB protein at 0.02% of TSP [82]. Mishra et al. [83] fused CTB with ubiquitin at N-terminal end increases the level further to 0.91% of TSP. In some cases, CTB fusions with target antigens have been used as a potent mucosal immunogen

and adjuvant because of its high binding affinity for the GM1-ganglioside receptor in mucosal epithelium. Then, it was shown to be functional by the GM1 binding assay [27, 84]. Recently, two CTB fusion proteins were expressed in tobacco and lettuce chloroplasts. Fusion proteins, containing CTB and the antigens AMA1 and MSP1 of malaria, were reported to be completely protected against cholera toxin (CT) challenge upon oral immunization. The expression level was 13.17% TSP and 10.11% TSP, respectively [85]. In another study, the fusion protein of CTB with fibronectin-binding domain (D2) of *Staphylococcus aureus*, a bacterium responsible for skin infections and bacteraemia, which may lead to life-threatening secondary infections such as endocarditis, showed the expression level as high as 23% TSP. The results revealed the induction of specific mucosal and systemic immune responses of fusion protein of the CTB-D2 in mice sera and faeces. The pathogen load in the spleen and the intestine of treated mice was significantly reduced in treated mice. Algae-based vaccination protected 80% of animals against lethal doses of *S. aureus*. Importantly, the alga vaccine was stable for more than 1.5 years at room temperature [86]. As described above, the fusion protein of CTB with proinsulin (CTB-pins, diabetes type 1) was achieved overexpression up to 72% of TSP in plastids [40].

The expression in most plastid transformation studies is higher than the expected threshold of 1% TSP which is considered as threshold to allow commercial or economical production [80]. Many other reports exist about high expression of vaccine antigens in plastids. For instance, Tregoning et al. [87] reported fairly high expression of a vaccine candidate TetC in tobacco plastids, accumulating up to 18–27% of TSP. Mucosal immunization of mice with the plastid-produced TetC induced protective levels of TetC antibodies [88]. Further, Tregoning and his colleagues reported that a single intranasal (i.n.) vaccination was as efficient as oral delivery, inducing high levels of activated CD4+ T cells and antitoxin antibody [87]. The high expression level of vaccine proteins such as vaccine virus envelope protein (A27L, Smallpox), human papillomavirus (L1), anthrax protective antigen (*pagA*), and HIV (p24-Nef) was up to 18% [89], 20–26% [90], 29% [18], 40% [91], respectively. Also, the immune responses of chloroplast made of these proteins were reported in the related studies. The chloroplast-made A27L protein was recognized by serum from a patient recently infected with a zoonotic OPV. Other characteristics included the ability to form oligomerize and the stability over a wide range of pH values. Hence, A27L protein could be a distinct advantage for the induction of intestinal secretory immunoglobulin A (IgA) following oral immunization [89]. The chloroplast-derived HPV16 L1 protein displayed conformation-specific epitopes and the ability of assembled into virus-like particles, highly immunogenic in mice after intraperitoneal injection, and neutralizing antibodies [90]. High antibody titers, especially IgG1 titers and IgG2a titers, were produced in plastid *pagA* gene expression protein treated mice. The antibodies from various groups were efficient in neutralizing the lethal toxin *in vitro*. When mice were challenged with *B. anthracis*, mice immunized with the protein imparted 60% and 40%

protection upon intraperitoneal and oral immunizations [10]. The largest cleavage product of HIV major gene-gag, p24, forms the conical core of HIV-1 viral particles and is the target of T-cell immune responses in both primary and chronically infected patients. The p24-Nef fusion and the p17/p24 protein effectively boosted T cell and humoral responses in mice [91, 92]. Therefore, chloroplast-derived vaccines have very promising and competitive potential for commercialization [80].

**4.2. Therapeutic Proteins and Antibiotics.** Recombinant therapeutic proteins are useful for treatment of various conditions such as genetic diseases that result in the production of an insufficient quantity or quality of a particular protein [79]. Plastid transgenic plant strategies have been used to demonstrate the production of many valuable human proteins, including insulin-like growth factor-1 (IGF-1), cardiotrophin-1, aprotinin, alpha-antitrypsin, and thioredoxin 1 [16]. Most therapeutic proteins also have competitive potential for commercialization. Among these proteins, the native and the optimized synthetic IGF-1 genes were expressed in *E. coli* and in transplastomic plants [93]. Expression of both genes was obtained only in transgenic tobacco plant line where the expression of IGF-1 reached up to 32% TSP using plastid light regulatory elements and under continuous illumination [79].

Both the rapid spread of antibiotic resistance and the stagnating discovery of new antibiotics have created an urgent need for alternative antimicrobials for clinical use. Recently, the use of the components of the phage that are needed to kill the bacterium would be a much-preferred strategy. These components, which possess highly efficient hydrolytic enzymes, are referred to as endolysins [78]. At the same time, plastid transformation has also been used as a novel strategy for large-scale, cost-effective production of next-generation antibiotics for topical and systemic treatments [75, 94, 95]. Three recent studies have explored and tested the potential of using chloroplasts as cheap factories for high-level production of endolysin-type protein antibiotics in tobacco chloroplasts: PlyGBS, Pal, and Cpl-1. Expression levels of the Pal, Cpl-1, and synthetic plyGBS gene in chloroplasts were 30%, 10%, and 70%, respectively [75, 95, 96]. In addition, two other potent disulphide-bonded antimicrobial peptides, protegrin-1 (PG1) and retrocyclin-101 (RC101), have also been investigated in transgenic tobacco plastids [94]. The expression levels of two peptides were as high as 26% and 38%, respectively. The antibacterial activity or antiviral infection of these antibiotic proteins was proved efficiently in plastid. Further details can be seen in recent two reviews by Bock and Warzecha [78] and Scotti et al. [16].

**4.3. Enzymes and Others.** Plastid transformation has been explored for the expression of various enzymes of biological and pharmaceutical importance. The gene for thermostable xylanase was expressed in the chloroplasts of tobacco plants [74]. Xylanase accumulated in the cells to approximately 6% TSP. Zymography assay demonstrated that the estimated

activity was  $421 \text{ U mg}^{-1}$  in crude TSP [97]. Recently, GH10 xylanase Xyl10B from *Thermotoga maritima* was expressed in tobacco chloroplasts and accumulated to high level of 13% TSP [98]. Enzymes such as endoglucanase from *Thermobifida fusca* and the endo- $\beta$ -1,4-glucanase E1 catalytic domain of *Acidothamus cellulolyticus*, which involved in biofuel production, were expressed in tobacco chloroplast and showed the expression level as high as 10.7% TSP [99] and 12% TSP [100], respectively. Endoglucanase Cel9A from *Thermobifida fusca* showed the expression level as high as 40% TSP [101]. Many other enzymes recently produced in plant chloroplast were described by Scotti et al. [16].

Also, chloroplasts have been used as bioreactors for production of biomaterial and amino acids. Normally, p-hydroxybenzoic acid (pHBA) is produced in small quantities in all plants. However, stable integration of the ubiC gene into the tobacco chloroplast resulted in hyperexpression of the enzyme and accumulation of this polymer up to 25% of dry weight [102, 103]. As we know, synthetic plastics are difficult to dispose off and continually accumulating nondegradable wastes have become a significant source of environmental pollution. Hence, biodegradable plastics seem to be a viable alternative to synthetic plastics [12]. As an alternative for efficient and inexpensive biomaterial production system, plants have also been engineered to produce polyhydroxybutyrates (PHBs) in the various plant cell compartments [1, 12]. Regenerated plants which an ecdysone analogue-based system was recently employed for induced gene expression in nuclear, contained up to 1-2% PHB (dry weight) in leaves after 6-8 weeks of induction [104]. Conversely, for the economic feasibility of transgenic plants-derived biodegradable plastics, accumulation of at least 15% of the tissue dry weight is required [105]. The biodegradable PHBs were expressed in plastids to increase the expression level. The expression levels ranging up to 40% of dry weight have been obtained [106]. Further, there is a need to aim at higher accumulation without any side effects on plants [12].

## 5. Current Problems and Future Prospects

Transgene expression from the plant's plastid genome has unique attractions to biotechnologists, including the plastids' potential to accumulate foreign proteins to extraordinarily high levels and the increased biosafety provided by the maternal mode of plastid inheritance, which greatly reduces unwanted transgene transmission via pollen. However, recent data [107] indicate that no transplastomic plant products have been licensed for this purpose as yet; even among all potential transgenic plant products, so far, only two have completed the regulatory processes for licensing: a recombinant single chain antibody to the hepatitis B surface antigen [108], and a Newcastle disease virus vaccine [109]. In addition, recombinant monoclonal antibodies for treatment of non-Hodgkin's lymphoma produced from a plant viral vector [110] were approved by the Food and Drug Administration for manufacturing, but the potential risks in their production and use, and the significant



investment of plant-based technology discouraged further progress [107]. This enormous potential notwithstanding, plastid transformation is still limited in its applications for the following issues to be resolved.

**5.1. Transformation Frequency and Transformation Vector.** As we know, plastid transformation was limited by low transformation frequencies in potato and other crops. Hence, a breakthrough in chloroplast genetic engineering of agronomically important species is a highly desirable goal. An approach, which used a modified regeneration procedure and novel vectors containing potato flanking sequence for transgene integration by homologous recombination, achieved efficiency up to one shoot every bombardment in potato transformation in large single-copy region of the plastome [111]. As vector delivery was performed by the biolistic approach, such efficiency corresponds to 15–18 fold improvement, and it is comparable to that usually achieved with tobacco. This represents a significant advancement toward the implementation of the plastid transformation technology.

Although chloroplast transformation technology has advanced significantly in the past two decades, the available plastid transformation vectors still lack several of the important functional features found in binary vectors [76]. First, only a limited number of genes of interest (GOIs) can be cloned into a single chloroplast transformation vector due to intrinsic multiple cloning site (MCS) limitations. Second, unlike binary vectors, which produce T-DNA capable of integrating into any nuclear genome sequence, chloroplast transformation vectors require a certain degree of homology with the plastid genome and, thus, may not be suitable for plant species with insufficiently conserved transgene integration sites [103]. To achieve high efficiency of transplastomic plant production, therefore, replacement of the homologous recombination sequences for each particular species/group of species through the time-consuming and laborious process of vector reconstruction may be required. Alternatively, the target plant itself can be converted into a “universal” recipient by integrating into its plastid genome-specific recombination sites, such as those for the phiC31 phage integrase, albeit also through lengthy experimentation [112].

**5.2. Regulation of Gene Expression.** The high expression of recombinant proteins within plastid-engineered systems offers a cost-effective solution for using plants as a bioreactor. In the expression of rHSA in tobacco chloroplasts, the yield of rHSA was increased 500-fold when compared with the expression level in nuclear transformation, reaching 11.1% of TSP [113]. However, this resulted in the formation of inclusion bodies, which needed a further renaturation or refolding process resulting in low recovery of rHSA after purification [50]. Although much of the forgoing discussion has implicitly assumed that maximizing the rate of translational elongation is unequivocally desirable, this is not entirely accurate. It has been suggested that too rapid translation may not allow for efficient “self” or chaperone-aided folding. So, slower codons or codon runs, perhaps

at protein domain boundaries, were strategically placed. This could maximize folding efficiency while maintaining a high overall translation rate [114]. In addition, constitutive expression of pharmaceutical proteins or unique metabolic pathways from the plastid genome can result in mutant phenotypes and/or severe growth retardation of transplastomic plants due to metabolite toxicities, interference with photosynthesis, or disturbance of the plastid endomembrane system. Recently, the approach based on an engineered riboswitch is established. This approach acts as a translational regulator of transgene expression in transformed plastids in response to the application of the ligand theophylline. The theophylline riboswitch offers a “plastid-only” solution to inducible gene expression from the chloroplast genome that does not require additional (nuclear or plastid) transgenes and thus should be widely applicable [115].

**5.3. Downstream Processing.** The main reason for the high cost of pharmaceutical protein production is purification of recombinant proteins. Also, this technology has not resulted in any product commercialization because problems in the protein purification still need to be solved as discussed later. Recently, a novel protein purification method is described carefully that do not require the use of expensive column chromatography [9]. Factually, this method is based on the inverse transition temperature ( $T_t$ ) of the polymers of elastin’s repeating VPGXG or GVGVP sequences. The inverse temperature transition property exhibited the phenomenon as temperature rises, the polymer collapses from an extended chain to a  $\beta$ -spiral structure with three VPGVG or GVGVP units per turn [9, 116]. Elastin as well as Elastin-like polypeptide (ELP) is well solvated and is highly soluble in aqueous solution below  $T_t$ . When the solution is heated and the  $T_t$  is reached, elastin become insoluble and form large micron-size aggregates that are visible to the naked eye [117]. Also, the environmental sensitivity and reversible solubility of ELPs are retained when an ELP is fused at the gene level with other proteins, and the activity of the ELP fusion protein is retained after cycling through the inverse phase transition [118]. This characteristic transition allows the recombinant ELP fusion protein to be isolated from the cell lysate by repeated steps of aggregation, centrifugation, and resolubilization without chromatography. Based on the previous description, a nonchromatographic method for protein purification was termed as inverse transition cycling (ITC). In ITC, a target protein or peptide is fused to the ELP at the gene level, expressed in *E. coli* or another expression system. After expression, the cells are lysed, and the cell debris is removed by centrifugation. The ELP fusion protein is then separated from soluble contaminants by triggering the phase transition of the ELP fusion protein [117, 119–121]. As for crystal structural and biochemical characterization, using proteases or self-cleaving ELP tags has been devised for purification of the tag-free recombinant proteins [120, 121]. So, ITC is both cost and time efficient because this purification method eliminates chromatography and scales up of this purification method is easy because it is not limited

by resin capacity. To date, ITC has been used to purify many protein ELP fusions, including cytokines, antibodies, and spider silk proteins from transgenic plants [120, 122]. Furthermore, this method will be used to purify protein ELP fusions in chloroplast expression system and to promote chloroplast transformation product commercialization.

Information about the stability of a protein can lead to a more thorough understanding of the mode of action as well as the effects of exposure to various conditions on the transgenic protein [123]. Protein stability represents more common causes of the lack of foreign protein accumulation in transgenic chloroplasts. Unfortunately, the rules governing protein stability in plastids and the identity of sequence motifs and/or structural motifs within the protein that confer stability or susceptibility to degradation are still a mystery. Unraveling the determinants of protein stability in plastids should potentially provide ways of stabilizing, otherwise, unstable recombinant proteins and, therefore, would be of enormous value to plastid biotechnology [78]. Recent studies have reported higher stability of the fused recombinant protein in chloroplast transformation. For example, thioredoxins (Trxs) are small ubiquitous disulphide proteins widely known to enhance expression and solubility of recombinant proteins in microbial expression systems. Sanz-Barrio and his colleagues reported that Trxs-HSA fusions markedly increased the final yield of human serum albumin (HAS, up to 26% of total protein) by higher HAS stability of the fused protein [124]. As discussed previously, RC101 and PG1, two important antimicrobial peptides, accumulate high expression levels by fusion with GFP to confer stability [94]. Additionally, disulphide bond formation is crucial for the biological activity of many therapeutic proteins. Alkaline phosphatase, whose activity and stability strictly depend on the correct formation of two intramolecular disulphide bonds, was expressed in tobacco chloroplast with the efficient formation of disulphide bonds [125]. Moreover, it is also envisaged that protein stability will change over time even with refrigeration [126]. The protein that the transgene encodes should be characterized to determine stability to pH, temperature, and chemical or biochemical agents involved downstream processing [123]. These need to be further studied on stability of recombinant protein in chloroplast transformation in the future.

As previous description, except proteins not requiring posttranslational modifications and nonglycosylated antibodies, it is not suitable to produce glycosylated recombinant proteins because chloroplasts are derived from ancient bacteria that are unable to do protein glycosylation [127], limiting the number of different proteins that can be produced using this system. In addition, plants are presently incapable of authentic human N-glycosylation to produce N-glycans that are essential for stability, folding, and biological activity of most therapeutic proteins. Thus, several glycoengineering strategies for the production of N-glycosylation in plants have emerged, including glycoprotein subcellular targeting, the inhibition of plant-specific glycosyltransferases, or the addition of human-specific glycosyltransferases [128, 129]. For example, based on the chloroplast proteome assay, Villarejo and his colleagues reported a chloroplast-located

protein which encoded a  $\alpha$ -carbonic anhydrase ( $\alpha$ -CA). The respective cDNA was denoted CAH1, and CAH1 was enriched in intact chloroplasts and the stroma fraction. CAH1 protein is not only taken up into the ER but is also glycosylated prior to being targeted to the chloroplast [130]. O-glycosylation is one of the most complex regulated posttranslational modifications of proteins and also one of the least understood [131]. Mucin-type (GalNAc-type) O-glycosylation is found in eumetazoan cells, but absent in plants and yeast. Recently, stably engineered mammalian-type O-glycosylation was established in transgenic plants, demonstrating that plants may serve as host cells for production of recombinant O-glycoproteins [132]. A large single-chain antibody against herpes simplex virus glycoprotein D was expressed and assembled correctly to form fully functional dimers by disulfide bond formation [34]. In addition, other posttranslational modifications, such as lipidation, can be achieved, as for the outer surface lipoprotein A of *Borrelia burgdorferi* expressed from the tobacco plastid genome [133]. These posttranslational modifications still poorly explored open interesting possibilities for plastid-based biotechnology and needed to further study in the future.

**5.4. Plant Species and Oral Delivery.** Although it has been achieved the development of improved selection/regeneration protocols and/or transformation vectors containing homologous flanking sequences in other plant species such as tomato, potato, eggplant, lettuce, and soybeans [18, 19, 134–137], routine chloroplast transformation is only possible in tobacco, which is inedible and highly regulated, being rich in toxic alkaloids. Hence, the extension of plastid transformation technology to other crops, especially those belonging to monocots, is still limited. In addition, to bring the plant to homoplastomy, where only transgenic genome-copies remain, further two or three rounds of regeneration on selective media are typically required [138, 139]. As with nuclear transgenics, it takes at least a year to generate production lines and to scale up. More experiments should be undertaken to move this technology toward practical utilization.

Some vaccine antigens and therapeutic proteins have only been expressed in tobacco. But tobacco is not edible and the addictiveness of nicotine also makes it unsuitable for oral delivery of therapeutic proteins [79]. Recently, lettuce (*Lactuca sativa* L.) chloroplast transformation has been developed. This system has been optimized and several therapeutic proteins have been expressed [140]. The level of expression in lettuce chloroplasts is similar to that in tobacco chloroplasts, and lettuce can be transformed as rapidly as tobacco. Thus, transformation in lettuce opens the door to practical oral delivery of chloroplast-expressed proteins. Chloroplast-derived therapeutic proteins, delivered orally via plant cells, are protected from degradation in the stomach, presumably because of bioencapsulation of the antigen by the plant cell wall [16]. To facilitate translocation of vaccine antigens or therapeutics from the gut lumen into the circulatory system, target proteins have been fused to

the CTB transmucosal carrier protein, which can bind to the epithelial receptor GM1 [141]. This approach has been widely applied to many orally delivered antigens, both for stable nuclear transgenics and for transplastomic approaches [93].

**5.5. Production Cost and Purification Cost.** Recombinant proteins may be expressed in bacteria, fungi and yeast, insect cells, mammalian cells, animals, or plants. The cost of the resulting product, especially downstream processing or purification cost has been described as the following. (1) The generation of recombinant proteins in bacterial system is faster and easier and thus allows for the easy progression to large-scale manufacturing [129]. Currently, the most widely used recombinant protein production systems are bacterial systems. However, the limitation of the presence of endogenously produced endotoxins and pathogens in *E. coli* is difficult and, therefore, costly to remove from target preparations and creates additional complexities [1]. (2) Foreign proteins production in yeasts and fungi offers the cost effectiveness and scaleup benefits of *E. coli* combined with the advantages of eukaryotic expression. Moreover, protein is expressed into the culture supernatant allowing faster and easier purification; as well, the purified protein contains less contaminating endotoxins as compared to the bacterially expressed counterparts [129]. However, they have a number of technical issues, such as the loss of plasmid and dramatic decrease in protein yield during large-scale production, which should be needed to resolve [1]. (3) Though insect cells have a number of advantages, several disadvantages exist. It has been shown that internal cleavage, at arginine- or lysine-rich sequences, is extremely inefficient and leads to improperly processed proteins. Furthermore, glycosylation capability is limited to only high mannose type [1]. (4) There are currently many established mammalian cell lines for the production of proteins. It should be noted, however, that the development of large-scale expression techniques is time consuming and requires high initial financial investment. Also, this system requires a nutrient media that is more complex than that of bacteria, fungi, or plants. So, the cost of the resulting product is quite substantial [1, 142]. (5) Transgenic technology has allowed for recombinant protein production in living animals such as rabbits, goats, pigs, and cows. Though the transformation of recombinant proteins in mammary glands has been showing a great promise, the production of recombinant protein in blood requires the use of high cost and complicated procedures. Moreover, the process of producing transgenic animals is labor, time, and cost intensive [1, 143]. (6) As for foreign proteins expressed in plants, it may be transiently transformed in nuclear, stably transformed in nuclear and chloroplast or plastid. More details are described as follows: (a) When recombinant proteins were generated by transient expression using RNA virus vectors or agrobacterium-mediated transient expression through Agroinfection, higher yields can generally be obtained. Recombinant proteins including antigen products have to be extracted and purified. Downstream processing of recombinant products is very expensive, amounting to more

than 80% of the total expense [7, 144]. (b) Product yields of recombinant proteins are usually <1% of total soluble proteins in nuclear transformation in plant. Hence, efficient protein purification from transgenic plants is a major challenge, and high impurity content in the feed streams. These drive up the costs of downstream processing [129, 144, 145]. However, if therapeutic proteins and vaccines are delivered orally, then edible transgenic plant offers the possibility of eliminating the need for expensive downstream protein purification and processing, especially in seed-based production [9, 144, 146]. (c) As described above, expression of a target gene from the chloroplast genome generally provides higher yields. The high expression of recombinant proteins within plastid-engineered systems offers a cost-effective solution for using plants as a bioreactor. As with nuclear transgenics, the cost for purification of therapeutic proteins and vaccines can be eliminated if they are orally delivered or minimized by the use of novel purification strategies [1, 147].

Recently, purification cost and production cost of bioreactor engineering for recombinant protein were assayed in bacterial, yeast, insect cells, mammalian cells, and plant cells. The low purification cost has been shown in plant cells, the medium purification cost has been shown in insect cells, and the high purification cost has been indicated in the three other cell lines. The results of the production cost of recombinant protein were different from that of the purification cost. The low production cost has been existed in bacterial (\$20–100/g), in yeast (\$20–100/g), in plant cells (\$50–100/g), respectively. The medium production cost has been exhibited in insect cells (\$50–200/g) and the high production cost exhibited in mammalian cells (\$1000–10000/g) [148]. In addition, according to the comparison with other expression systems, the overcost of plant-based production platforms is as low as that of bacteria and yeast, and the purification cost is as high as that of bacteria and yeast [149].

**5.6. Other Problems.** Chloroplast genetic engineering is considered a “plant safe” strategy. However, few reports indicated that pleiotropic effects of vaccine antigens exhibited some detrimental effects, such as male sterility, yellow leaves, and stunted growth of transplastomic plants, especially in the expression of *phaA* gene in chloroplasts [80]. Except the limitations described above, there are many confrontations that lie in issues that still need to be addressed and solved: these apply mainly for evaluation of the transplastomic plants, the efficacy of plastid derived vaccines, the regulatory issues development of plastid transformation system for edible plant species and marker excision from transplastomic plants. Many of these challenges are discussed in detail in the previous and recent reviews [80, 107, 150].

Based on the previous description, edible transgenic plant tissue will eliminate the downstream processing or purification cost. Production of recombinant proteins in plant is no need to maintain the cold chain as the plant parts expressing the vaccine or plant extracts can be stored and transported at room temperature [9]. Nevertheless, due to

the perishable nature of the fruits and vegetables they require immediate processing to avoid postharvest losses (20–25%) [151]. So, nonthermal processing of fruit and vegetable has been revealed as a useful tool to extend their shelf-life and quality as well as to preserve their nutritional and functional characteristics [152]. In addition, most batches of feed or food derived from genetically modified (GM) plants for the safety study should be kept as cold as possible, depending on the storage time needed [153]. When tobacco and lettuce chloroplasts were transformed with the CTB fused with human proinsulin, old tobacco leaves and old lettuce leaves accumulated proinsulin up to 47% of total leaf protein (TLP) and 53% TLP, respectively. Even in senescent and dried lettuce leaves, accumulation was so stable that up to ~40% proinsulin in TLP was observed. This may promote to facilitate their processing and storage in the field [154]. Typically, intake of a protein is estimated by considering actual expression levels in consumed tissues (i.e., fruit or grain versus leaves) and by considering a comprehensive evaluation of food consumption practices of the population [123]. The exposure levels are based on the concentrations of the transgenic protein likely to be encountered in the human diet. According to the OECD Guideline, a single-dose and repeated-dose toxicologies need further studied to predict human exposure [123]. Based on the previous information, except tobacco, recombinant proteins were mainly explored in a number of plant species, including a few vegetables species. Therefore, the shelf life and dose of edible transgenic plants, especially in plastid transformation, are rarely studied. Further research will be necessary for exploring food or feed demands on edible plants.

## 6. Conclusions

The plastid transformation offered a good platform of foreign gene expression in high plants. In the joint efforts of researchers all over the world, plastid transformation in plants has made considerable progress. To date, more than 50 different transgenes have been stably integrated and expressed via the plant plastid genome to confer important agronomic traits, as well as to produce industrially valuable biomaterials and therapeutic proteins. The ability to engineer chloroplast as an alternative site for the expression of foreign genes, proteins, reactions, and products has gained prominence relatively recently. Considering the recent scientific and technological developments in plastid transformation technology, such as the marker gene elimination systems, the possibility to induce gene expression, the development of novel purification method, and the selection of novel regulatory sequences for expression in chloroplasts, it can be predicted that in the next future the plastid transformation approach will be applied to a larger set of species and for a wider range of purposes.

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