Electronic Journal of Biotechnology 45 (2020) 1–9

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Original Research Article

Transient expression of a green fluorescent protein in tobacco and maize chloroplast

Sigifredo Arévalo-Gallegos [a], Hugo Varela-Rodríguez [a], Héctor Lugo-Aguilar [a], Tania S. Siqueiros-Cendón [a], Blanca F. Iglesias-Figueroa [a], Edward A. Espinoza-Sánchez [a], Gerardo A. Aguado-Santacruz [b], Quintín Rascón-Cruz [a,*]

[a] Laboratorio de Biotecnología I, Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Circuito Universitarios s/n, Nuevo Campus Universitario, C.P. 31125, Chihuahua, Chihuahua, México
[b] Instituto Tecnológico de Roque, Carretera Celaya-Juventino Rosas Km. 8, C.P. 38110, Celaya, Guanajuato, México

ARTICLE INFO

Article history:
Received 5 September 2019
Accepted 28 January 2020
Available online 4 February 2020

Keywords:
chloroplast genome
Chloroplast transformation
Green fluorescent protein
Maize
mgfp5 gene
Plastid transformation
Recalcitrant crops improvement
Tobacco
Transgenes
Transient expression
Zea mays

ABSTRACT

Background: Maize is one of the most important crops worldwide and has been a target of nuclear-based transformation biotechnology to improve it and satisfy the food demand of the ever-growing global population. However, the maize plastid transformation has not been accomplished due to the recalcitrant condition of the crop.
Results: In this study, we constructed two different vectors with homologous recombination sequences from maize (*Zea mays* var. LPC13) and grass (*Bouteloua gracilis* var. ex Steud) (pZmcpGFP and pBgcpGFP, respectively). Both vectors were designed to integrate into *rrn23S*/*rrn16S* from an inverted repeat region in the chloroplast genome. Moreover, the vector had the *mgfp5* gene driven by P*rrn*, a leader sequence of the *atpB* gene and a terminator sequence from the *rbcL* gene. Also, constructs have an *hph* gene as a selection marker gene driven by P*rrn*, a leader sequence from *rbcL* gene and a terminator sequence from *the rbcL* gene. Explants of maize, tobacco and *Escherichia coli* cells were transformed with both vectors to evaluate the transitory expression–an exhibition of green and red fluorescent light under epifluorescence microscopy. These results showed that both vectors were expressed; the reporter gene in all three organisms confirmed the capacity of the vectors to express genes in the cell compartments.
Conclusions: This paper is the first report of transient expression of GFP in maize embryos and offers new information for genetically improving recalcitrant crops; it also opens new possibilities for the improvement in maize chloroplast transformation with these vectors.
How to cite: Arévalo-Gallegos S, Varela-Rodríguez H, Lugo-Aguilar H, et al. Transient expression of a green fluorescent protein in tobacco and maize chloroplast. Electron J Biotechnol 2020;44. https://doi.org/10.1016/j.ejbt.2020.01.008

1. Introduction

Genetic engineering plays an important role in the improvement of crop plants through the incorporation foreign genes and, during the past two decades, has revolutionized agriculture. Improvement in several crops has been achieved by nuclear transformation. However, this technique suffers from several disadvantages such as position effects, gene silencing, epigenetic effects, non-contention of transgenes, and low levels of transgene expression which can be solved by chloroplast engineering [1,2,3,4,5,6,7]. Plastid transformation was achieved first in the unicellular green alga *Chlamydomonas reinhardtii* [8], followed by stable plastid transformation in tobacco. It has been reported that currently, homoplasmic plants of 18 species of flowering plants have been obtained via stable plastid transformation [9].

Several transgenes of industrial value have been introduced successfully in rapeseed '*Brassica napus*', eggplant '*Solanum melongena*', bitter melon '*Momordica charantia*', potato '*Solanum tuberosum*', tomato '*Solanum lycopersicum*', lettuce '*Lactuca sativa*', cauliflower '*Brassica oleracea*', poplar '*Populus alba*', carrot '*Daucus carota*', cotton '*Gossipyum hirsutum*, soja '*Glycine max*' and rice '*Oryza sativa*' [9,10,11,12,13,14,15,16,17,18,19,20,21,22,23] with expression

* Corresponding author.
E-mail address: qrascon@uach.mx (Q. Rascón-Cruz).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.01.008

levels up to 70% of total soluble protein due to the existence of several copies of plastid genome per cell [24,25].

However, although this technology is highly promising, none of the agronomically important monocot cereal crops such as wheat (*Triticum aestivum)* and maize (*Zea mays)* have been reported to obtain the homoplastomic plants via plastid transformation [9]. Although there are reports of plastid transformation of calli in maize [26], this kind of plants seems to be recalcitrant to plastid transformation, and plastid transformation has been established only in a few monocotyledonous crops [27]. In this sense, Khan and Maliga [28] reported stable transformation in rice (*Oryza sativa* cv. Taipei 309), but the transgene transmission to subsequent generations could not be verified. Nevertheless, later, japonica rice (*Oryza sativa* L. cv. Hwa-Chung) was transformed with *aadA* and *sgfp* genes inserted in *trnI-trnA* inverted repeat regions achieving the inheritance of plastid-expressed transgenes to the progeny of a transplastomic cereal crop, although with lower transformation efficiency and heteroplasmy [16]. Wang et al. [9] transformed japonica rice line 19 with the *smGFP* gene and *htp* select marker genes inserting them into *rrn16*/*rrn23* from inverted repeat region obtaining homoplasmic plants. Also, in 2012, transformation of wheat (*Triticum aestivum* L. cv. Bobwhite) was reported, but the results could not be confirmed [29].

Usually for transformation analysis, a selection of marker genes has been used. For example, the *aadA* gene that encodes for aminoglycoside 3' acetyltransferase that confers resistance to spectinomycin [5], and the *hph* gene that encodes to hygromycin B 4-O-kinase and confers resistance on plant cells by detoxifying hygromycin B. The latter has proved to be more effective in a selection of several species of plants, including monocots [30,31] even more than the gene *npt*II [32]. The genetic improvement also uses reporter genes such as green fluorescent protein (GFP). GFP is characterized by low molecular weight, stable structure, easy detection, and nontoxicity. It is a chromophore that absorbs blue or ultraviolet (UV) light and fluoresces green [33,34,35,36]. Thus, GFP proves useful as the fluorescent gene product to be introduced in living cells for characterizing genes and proteins of interest in a transformed cell or a tissue without subjecting the experimental material to destructive, prolonged, or lethal procedures of analysis, as such, transient gene expression is widely used [28,37].

Transient transformation, by definition, results in temporary alterations in gene expression, and this approach is often useful as rapid analysis of transient gene expression can provide valuable information on the functionalities of the coding region or gene components like promoters, introns or terminators that affect the timing or intensity of expression. Although these approaches have limitations, they reduce the duration of analysis to days, instead of months or years [38]. Currently, the transplastomic technology has been extended to agronomically important crops [39,40] for the development of transplastomic cereal crops by the improvement of the genetics of plants, which is perceived as a challenge. In this study, we report for the first time the efficient transient expression of GFP in embryos of maize and tobacco leaves using transformant chloroplast vectors that can express transgenes in a stable form for improving agronomic traits in maize.

2. Materials and methods

2.1. Construction of chloroplastidic vectors

PCR amplification was used to obtain the *rrn16S:trnI:trnA:rrn23S* regions of chloroplast genome from *Zea mays* (tropical variety 'LPC13' line, provided by Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, México) and *Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Steud (collected locally from the prairies at Chihuahua, México). The primers were designed with GenBank sequence from maize (NC_001666) Fw-5'ATCCTCTCCTTCCTCCGGCT3' to *rrn16S* and Rv-5'AGTCGCCGGCTCATTCTTCA3' to *rrn23S*. The PCR was performed at 3 min of denaturing at 94°C, followed by 30 cycles of amplification (45 s at 94°C, 45 s at 63°C, 2 min at 70°C), and a final extension of 6 min at 70°C. These primers were amplified 4,977 bp from *Z. mays* and 4,494 bp from *B. gracilis*, and the PCR products were inserted into *Eco*RV-digested pMOS*Blue*® vector to obtain pTLPC135kb and pTBg5kb chloroplastidic vectors; chloroplast sequences were previously reported by our research group and deposited in NCBI under EU282003 (for *Z. mays* and *B. gracilis,* respectively) to allow homologous recombination in inverted repeats from these chloroplast genomes.

On the other hand, an expression cassette of 2.786 kb was synthesized by Epoch Life Science (Missouri, TX, USA) flanked by *Aat*II sites and cloned in pBSK–GS51885 vector. This cassette was obtained and inserted in the *Aat*II site from expression vectors pTLPC135kb and pTBg5kb. The cassette contained the reporter GFP thermotolerant variant encoded by '*mgfp5*' gene and the selectable marker hygromycin B 4–O–kinase encoded by *hph* gene (both from pCAMBIA1302 'GenBank: AF234298'). These sequences were designed *in silico* using Gene Designer v2.0.176 software with undesirable restriction sites eliminated by silent mutation. The coding sequence of the *mgfp5* (756 bp) was synthesized with a leader sequence and DB box from *atpB* gene upstream, whereas the coding sequence of *hph* gene (1,026 bp) was synthesized with a leader sequence and DB box from *rbcL* gene upstream. Both DNA sequences were obtained from the plastidial genome of *Nicotiana tabacum* (GenBank: NC_001879). The two genes were driven by *rrn16S* promoter (P), a Shine-Dalgarno (SD) sequence and a terminator (T) from *rbcL* gene for mRNA stabilization and to enhance gene translation [41]. Both promoter and terminator were synthetic and were obtained from sequences reported by Kuroda and Maliga [42]. The sequences were designed to reflect the native codon usage of the chloroplast genome.

2.2. Plastid transformation

Young tobacco leaves and embryos from young maize seeds were used. Tobacco leaves (*Nicotiana tabacum* var. Petite Havana) were obtained from plants grown under sterile conditions on RMOP culture medium (MS salts with 4.3 g/L, sucrose 30 g/L, myo–inositol 100 mg/L, thiamine–HCl 1 mg/L, 6–BAP 1 mg/L, 1–NAA 0.1 mg/L, Gelrite® 3 g/L, pH 5.8) four weeks before bombardment. On the other hand, immature embryos from maize seeds of 1-2 mm were placed in N6E medium culture (N6E salts with 4 g/L, 2,4D 2 mL/L, L–proline 2.8 g/L, sucrose 30 g/L, hydrolyzed casein 100 mg/L, myo–inositol 100 mg/L, Gelrite® 3 g/L, $AgNO_3$ 25 μM, pH 5.8) four days before bombardment. For maintenance and propagation of plasmids, *Escherichia coli* strain DH5α and Mach1™–T1R (Invitrogen®, Carlsbad CA, USA) was used and grown in LB medium supplemented with 100 μg/mL ampicillin/hygromycin B. Plasmid DNA was obtained using GenElute HPTM Plasmid Midiprep Kit® (Sigma-Aldrich). Tungsten particles of 0.7 μm (Bio-Rad®) coated with 0.1 μg of DNA per microliter of particles were used for the transformation of immature maize embryos [43], and tobacco leaves by bombardment according to Svab and Maliga [44] and Lutz et al. [41]. System PIG (Particle Inflow Gun) constructed in the laboratory was used for the bombardment at 80 psi from 17 cm shooting distance.

To contrast the expression of GFP, *E. coli* Mach1™–T1 colonies were transformed with the pBSK-GS51885 and pCAMBIA1302 vectors containing the *mgfp5*/*hph* cassette and cultivated in LB medium supplemented with 100 μg/mL of ampicillin/hygromycin B and grown for 12 h; resistant clones were recruited by 9 h at 37°C in liquid LB medium until 0.7 O.D. at 600 nm.

2.3. PCR screening

Plasmid DNA was extracted from bacterial samples with NucleoSpin® Extract II (Macherey–Nagel GmbH & Co. KG). PCR was performed using specific primers: *mgfp5* gene Fw-5'GCTAGCATGGTAGATCTGACCAGTAA AGGAG3', Rv-5'TCTAGAGGTGGCTTCATTTGTATAGTTCATC3' and *hph*

gene Fw-5'GCTAGCATGAAAAAGCCTGAACTCACC3', Rv-5'TCTAGACTATT TCTTTGCCCTCGGAC3', under the following conditions: 3 min of denaturing at 94°C, followed by 30 cycles of amplification (45 s at 94°C, 45 s at 63.5°C, 2.15 min at 70°C) and a final extension of 6:45 min at 70°C.

2.4. Detection of GFP by epifluorescence microscopy

Olympus BX41 microscope with U–MSWB2 filter was used for the expression analysis of GFP. Fresh cultures from transformed bacterial cells were concentrated by centrifugation at 2,000 rpm for 4 min and submitted to analysis, whereas vegetal explants were observed 20 h after bombardment. The exposition used was with a maximum time of 30s at 5X and 10X.

3. Results

3.1. Vector construction with mgfp5 and hph genes

The two vectors had *rrn23S*/*rrn16S* sequences for integration via homologous recombination in the inverted repeat region (IRA/IRB) of the chloroplast genome (including the *trn*A and *trn*I sequences) obtained by PCR amplification. In both constructions, the genes were integrated into the *trn*I – *rrn16S* interspaced region. The gene *mgfp*5 was synthesized with a promoter from *rrn16S*, *a* leader sequence and DB box from the wild-type *atpB* gene as 5'*UTR* and a 3'*UTR* from the *rbcL* gene. On the other hand, the *hph* gene was synthesized with a promoter from *rrn16S*, a leader sequence and DB box from the wild-type *rbcL* gene as 5'*UTR* and a 3'*UTR* from the

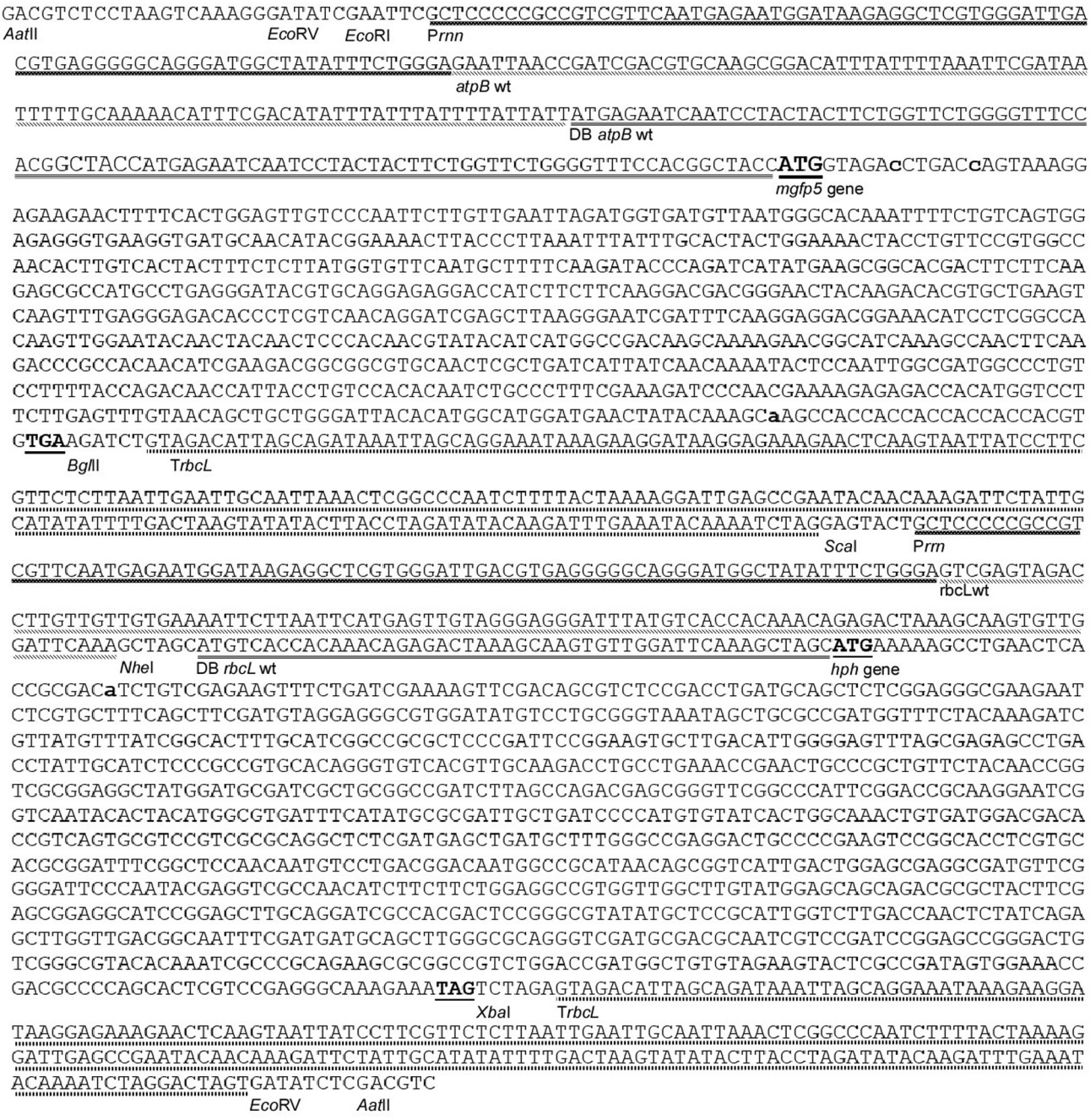

Fig. 1. Synthetic cassette with the both *mgfp5* and *hph* genes driven by P*rrn* and T*rbcl* cloned into pBSK-GS51885. The genes have leader sequence *atpB* wt and DB *atpB* wt upstream from the *mgfp5* gene; also, leader sequence *rbcL* wt and sequences DB *rbcL* wt upstream of the *hph* gene. In continuous line, the different regulatory regions are shown, and the start and end codons are indicated by underline; also, in lowercase, the point mutations made in the sequence are displayed.

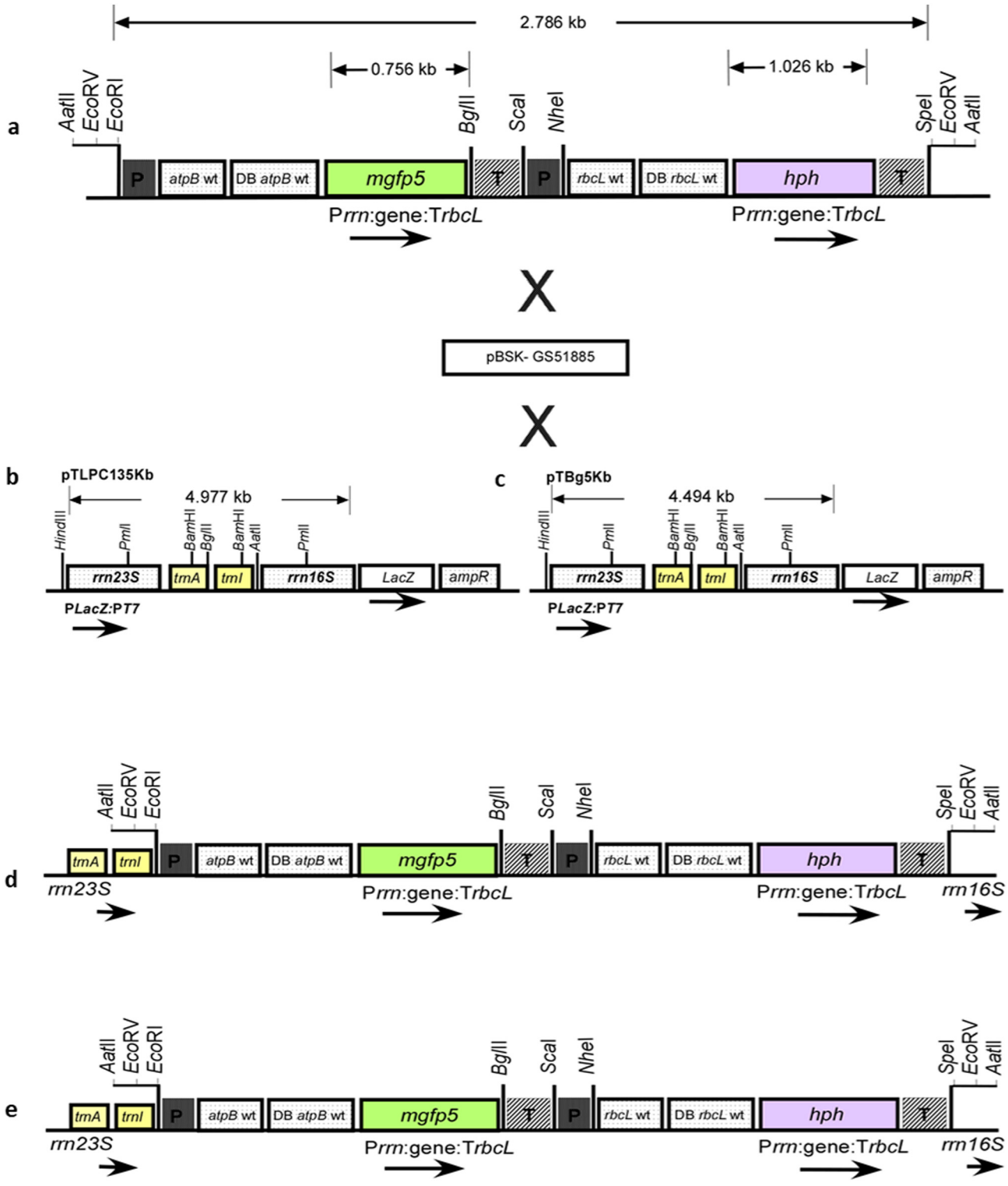

Fig. 2. Vector design. (a) Synthetic cassette with both *mgfp5* and *hph* genes driven by P*rrn* and T*rbcL* cloned into pBSK-GS51885. (b,c) Chloroplastidic vector with homologous recombination regions from *Z. mays* and *B. gracilis*, respectively. (d) pZmcpGFP plasmid obtained from pTLPC135kb. (e) pBgcpGFP plasmid obtained from pTBg5kb.

rbcL gene (Fig. 1 and Fig. 2a). In this research, we developed two plastid transformation vectors. The *mgfp5* and *hph* genes were designed, synthesized and cloned in the pTLPC135kb and pTBg5kb backbone vectors (Fig. 2b and c) to yield the final vectors pZmcpGFP and pBgcpGFP (Fig. 2d and e).

3.2. Chloroplast and bacterial transformation

3.2.1. PCR analysis

E. coli Mach1™–T1 colonies were transformed with the pZmcpGFP and pBgcpGFP plasmids to integrate the *mgfp5*/*hph* genes, and their

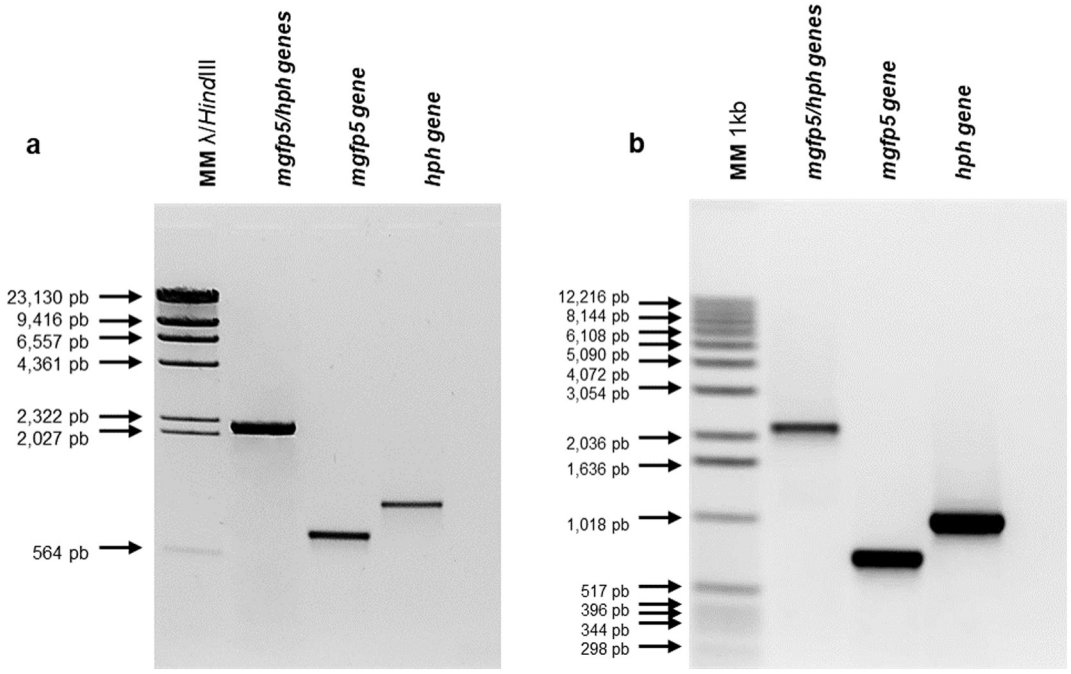

Fig. 3. PCR analysis. (a) pZmcpGFP, (b) pBgcpGFP. A fragment of ≈2.2 kb was amplified with the *mgfp5/hph* jointly. The amplification of *mgfp5* gene showed a fragment of ≈0.7 kb, whereas the *hph* gene showed ≈1.0 kb. Both construction showed fragments of the length of the genes of interest.

expression was evaluated. Both plasmid DNA were tested by PCR screening to confirm the integration of the genes of interest. The plasmid clones showed the ≈0.70 kb and ≈1.0 kb amplification of *mgfp5* and *hph* genes, respectively. Also, the gene integration was confirmed by the fragment of ≈2.20 kb obtained from both genes (Fig. 3).

3.2.2. GFP analysis for epifluorescence microscopy in bacterial cells

Bacterial cells were transformed and grown in the presence of selection marker and recruited and analyzed for GFP activity. Plasmid pBSK–GS51885 was used as a positive control, and several fluorescent points were detected in the transformed clones, indicating the presence of the gene expression (Fig. 4a). On the other hand, transformed clones with pZmcpGFP and pBgcpGFP plasmids also showed heterogeneous fluorescent points (Fig. 4b and c), whereas the nontransformed clones did not show fluorescent points (Fig. 4d) indicating that the transcription, folding, and processing was successful.

3.2.3. GFP analysis for epifluorescence microscopy in vegetal cells

Young tobacco leaves and immature embryos from young maize seeds were transformed by biolistic with the pCAMBIA1302, pZmcpGFP and pBgcpGFP vectors. Sixteen hours after the bombardment, the tobacco leaves showed irregularly distributed yellow fluorescent points of variable intensity. Leaves bombed with pCAMBIA1302 vector showed intense yellow fluorescent points of similar size (Fig. 5a), which indicated that a small percentage of cells was transformed. In leaves bombarded with chloroplastidic vectors (pZmcpGFP and pBgcpGFP), the fluorescent expression was localized in defined zones on the inner peripheries of cellular plasmalemma (Fig. 5b and c), whereas non-bombarded tissue was devoid of fluorescent points (Fig. 5d).

On the other hand, immature embryos from young maize seeds bombarded with the aforementioned constructed vectors showed similar green fluorescent points in scutellum. The cells bombarded with pCAMBIA1302 vector exhibited intense fluorescence in irregular zones and, being diffused, their number could not be counted (Fig. 6a). On the other hand, *Z. mays* embryos bombarded with pZmcpGFP and pBgcpGFP plasmids showed differentiated fluorescent points concentrated in zonal clusters (Fig. 6b and c), whereas negative controls did not exhibit fluorescent points (Fig. 6d and e). Tobacco leaves showed red fluorescent color, whereas immature embryos from young maize seeds showed green fluorescence (Fig. 5 and Fig. 6).

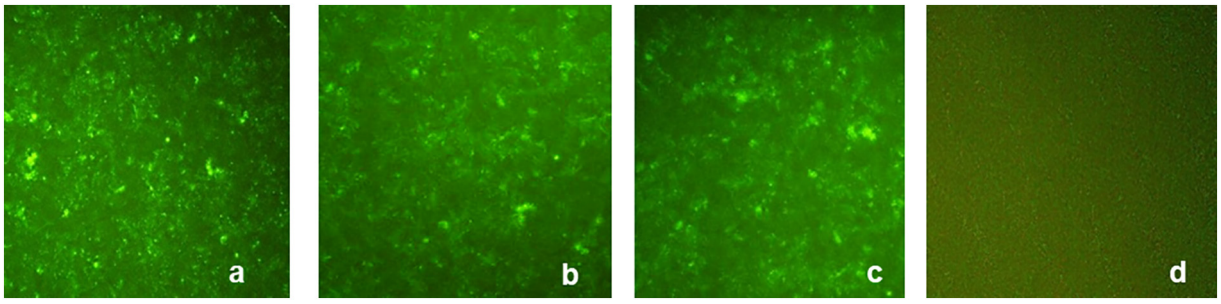

Fig. 4. Expression analysis of *mgfp5* and *hph* in *E. coli* Mach1™–T1 growth in LB medium supplemented with 100 μg/mL ampicillin/hygromycin B. (a) Transformed bacterial cells with the pBSK-GS51885 plasmid. (b) Transformed bacterial cell with the pZmcpGFP plasmid. (c) Transformed bacterial cells with the pBgcpGFP plasmid. (d) Non-transformed bacterial cells (observations at 10X).

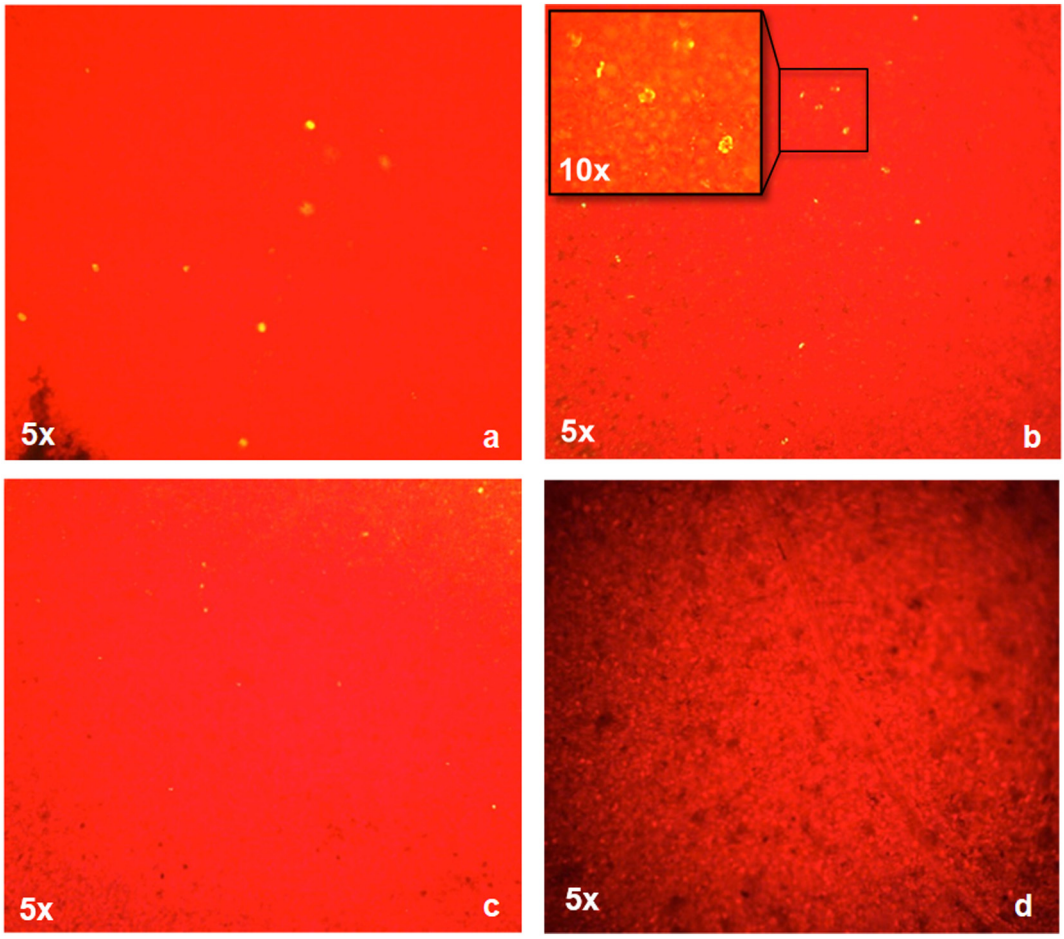

Fig. 5. Transient expression analysis in bombed tobacco leaves (*Nicotiana tabacum* L. var. Petit Havana). (a) Bombed leave from tobacco with pCAMBIA1302. (b) Bombed leave from tobacco with pZmcpGFP vector. (c) Bombed leave from tobacco with pBgcpGFP vector. (d) Bombed leave without plasmidic DNA.

4. Discussion

As a platform of genetic improvement and expression of valuated proteins, plastid transformation technology has been widely accepted an attractive alternative in the field of genetic engineering. Currently, the transplastomic crops are promising to improve food production. Though heteroplasmatic monocot plants like rice have reportedly been transformed by different groups [9,16,28], the high interest in extending plastid transformation to other major cereal crops by the application of this technology is hampered by the recalcitrance of these crops to plastid transformation. Therefore, this technology is limited to a few dicotyledonous plants, mainly using green leaves that contain developed chloroplasts because of the difficulty in expression of transgenes in non-green plastids and the inability to generate homoplastomic plants via subsequent rounds of regeneration using leaves as explants [10,13,16,27,29]. Furthermore, until today, maize plastid transformation has not been reported [9] and one study on wheat transformation was retracted [45] reinforcing the idea of the recalcitrance of cereals to the plastids transformation.

In the present study, we report the transient transformation of *Zea mays* and *Nicotiana tabacum* chloroplast by using the biolistic technique and contrasted with *E. coli*. The vectors used for chloroplast transformations were constructed in our laboratory. Both vectors had the *rrn16S:trnI:trnA:rrn23S* regions as homologous recombination sites, one from *Z. mays* and the other one from *B. gracilis,* such as were previously reported for other chloroplast regions [4,9,11,46,47,48]. Extended sequence and structure homology have resulted in a comparative analysis of *rrn16S:trnI:trnA:rrn23S* regions as between *Zea mays* and *Bouteloua gracilis* [49].

The vectors contain the *mgfp5* gene, which encodes for the GFP protein and has been extensively used as a reported marker for visualization of gene expression in both nuclear and chloroplast transformation in plants [50,51,52,53]. The *mgfp5* gene is driven by P*rrn* as a promoter, L*atpB* as 5'UTR, and T*rbcl* as 3'UTR [1]. The vector also contains the *hph* gene as a selection marker [29] controlled by the promoter P*rrn* and L*rbcL* sequence as 5' *UTR* and a T*rbcl* as 3'UTR.

With the constructed chloroplast vector, it is possible to report the subsequent transient expression in tobacco leaves and immature embryos from maize. In general, the selectable marker genes used in this research are driven by strong constitutive promoters [54]. It has been reported that the use of 5'UTR and 3'UTR from *rrn* and *rbcL* is the most critical factor for increasing heterologous protein expression in tobacco chloroplast [55,56,57,58]. This work has now confirmed that the use of those synthetic sequences could also be used to induce the gene expression in young embryos extracted from maize seeds even though they have been reported as being not rich in proplastids [16,59].

Although there are verified reports of GFP expression in transformed clones of forage crops [16,45,60,61,62] there are a few studies related to GFP expression in chloroplast from maize. According to Chiu et al. [52], high level expression is important to yield good fluorescent signals. In

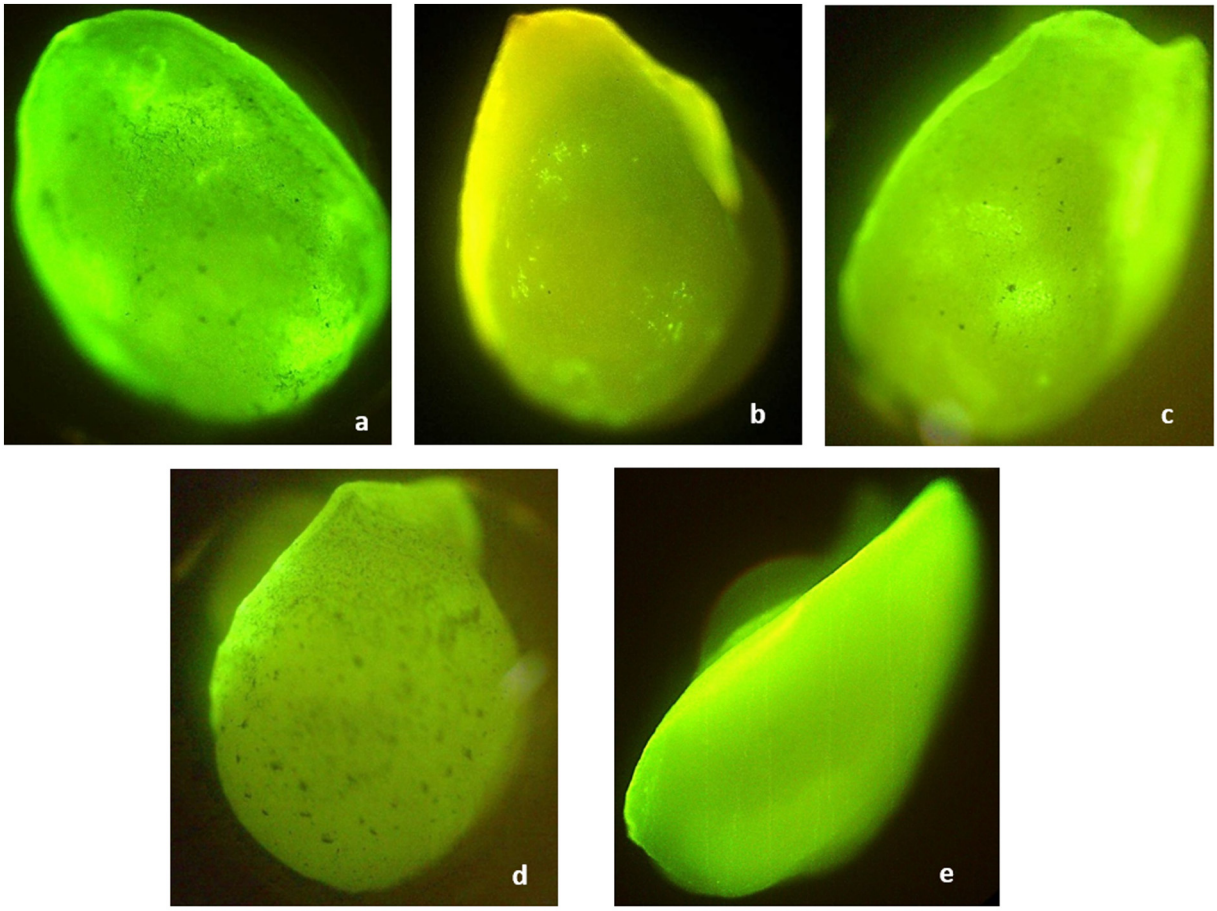

Fig. 6. Transient expression analysis in bombed immature embryos from young corn seed (*Zea mays* L.). (a) Embryos bombed with pCAMBIA1302 plasmid. (b) Embryos bombed with pZmcpGFP vector. (c) Embryos bombed with pBgcpGFP vector. (d) Embryos bombed without plasmidic DNA. (e) Embryos nonbombed (observations at 5X).

this study, we achieved the transient expression in both tobacco and maize verified by fluorescence emission by using two constructed chloroplast vectors. The fluorescence emitted in putative chloroplast expression was observed in the localized regions of synthesis and chloroplastidic confinement in the cell as would be expected, whereas in nuclear transformation, the fluorescence was observed as irregular and diffuse emissions as would be expected from cytoplasmic synthesis. Similar results were obtained in the nuclear expression of GFP driven by globulin-1 [63]. The putative chloroplastidic expression is supported by the high promoter *rrn* in chloroplast vectors, which requires chloroplastidic polymerase PEP to be absent in cellular core [64]. A difference of red and green autofluorescence was observed. Tobacco leaves showed red autofluorescence due to the presence of chlorophyll *a* and *b,* whereas embryos from maize emitted autofluorescence green due to the presence of flavin and absence of chlorophyll [65,66]. Although the microscopic analysis indicated transitory GFP expression in the gene and the effectiveness of transformation in tobacco and maize tissue, the experimental design does not ensure in any way the integration of the transgenes *mgfp5* and *hph* into the plastome. Further studies are needed to investigate stable integration of the transgenes in these tissues.

Despite the difficulty in the transformation in cereal crops [10], the increased understanding of gene expression in green and non-green plastids, improvements in plant regeneration systems and gene transfer methods will facilitate the extension of the plastid transformation technology to a larger set of plant species [15,27]. Though currently, there are no reports of stable chloroplast transformation in maize, this study supports the development of novel tools to aid in chloroplast gene expression to be used in the development of biotechnological crops.

Acknowledgments

We thank MC Amado J. Sardiña Peña for compiling manuscript information.

Financial support

Héctor Lugo-Aguilar, Hugo Varela-Rodríguez and Blanca F. Iglesias-Figueroa thank for grants from Universidad Autónoma de Chihuahua and CONACYT México.

Conflict of Interest

The authors declare that they have no conflict of interest

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