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Human Granulocyte Colony-Stimulating Factor (hG-CSF) Expression in Plastids of *Lactuca sativa*

Mehdi Sharifi Tabar[1], Ali Akbar Habashi[*2] and Hamid Rajabi Memari[3,4]

[1]*Molecular Systems Biology Group of Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran;* [2]*Dept. of Tissue Culture and Cell Transformtion, Agriculture Biotechnology Research Institute of Iran (ABRII), Seed and Plant Improvement Institutes Campus, Karaj, Iran;*
[3]*Biotechnology and Life Science Centre, Shahid Chamran University of Ahvaz, Ahvaz, Iran;*
[4]*School of Agriculture, Shahid Chamran University of Ahvaz, Ahvaz, Iran*

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ABSTRACT

Background: Human granulocyte colony-stimulating factor (hG-CSF) can serve as valuable biopharmaceutical for research and treatment of the human blood cancer. Transplastomic plants have been emerged as a new and high potential candidate for production of recombinant biopharmaceutical proteins in comparison with transgenic plants due to extremely high level expression, biosafety and many other advantages. **Methods:** *hG-CSF* gene was cloned into pCL vector between prrn16S promoter and TpsbA terminator. The recombinant vector was coated on nanogold particles and transformed to lettuce chloroplasts through biolistic method. Callogenesis and regeneration of cotyledonary explants were obtained by Murashige and Skoog media containing 6-benzylaminopurine and 1-naphthaleneacetic acid hormones. The presence of *hG-CSF* gene in plastome was studied with four specific PCR primers and expression by Western immunoblotting. **Results:** *hG-CSF* gene cloning was confirmed by digestion and sequencing. Transplastomic lettuce lines were regenerated and subjected to molecular analysis. The presence of *hG-CSF* in plastome was confirmed by PCR using specific primers designed from the plastid genome. Western immunoblotting of extracted protein from transplastomic plants showed a 20-kDa band, which verified the expression of recombinant protein in lettuce chloroplasts. **Conclusions:** This study is the first report that successfully express *hG-CSF* gene in lettuce chloroplast. The lettuce plastome can provide a cheap and safe expression platform for producing valuable biopharmaceuticals for research and treatment. *Iran. Biomed. J. 17 (3): 158-164, 2013*

Keywords: Plastid, Human granulocyte colony-stimulating factor (hG-CSF), Biolistics, Gene targeting

INTRODUCTION

Human granulocyte colony-stimulating factor (hG-CSF) is a proliferation, differentiation, survival and activation factor for hematopoietic cells [1]. hG-CSF consists of various products, which are derived from different tissues. hG-CSF stimulates the bone marrow to produce granulocytes by releasing them into the blood. It also stimulates the proliferation, differentiation, survival and function of neutrophil precursors and mature neutrophil introduction. The mature hG-CSF is a 19.6-kDa glycoprotein containing 174 amino acids [2, 3]. hG-CSF is produced in different platforms, such as *Escherichia coli* [3], yeast [4], transgenic plant [5] and mammalian cells [6] and in some cases, it is applied for clinical uses. Recombinant form of hG-CSF is successfully used to treat cancer patients suffering from chemotherapy-induced neutropenia and help them to accelerate recovery process [7].

In 1986, it was shown that tobacco plants and sunflower calluses could express recombinant human growth hormone [8]. Since then, a diverse range of plant systems has been used for the production of biopharmaceuticals [9-13]. The first plant-made biopharmaceutical was approved by USA Food and Drug Administration on May 2012 and produced in carrot for treatment of type 1 Gaucher's disease [14].

Plants provide opportunities as a safe method of delivery of recombinant proteins for therapeutic use, easy storage and distribution. Furthermore, there are fewer concerns about product safety and public

*Corresponding Author; E-mail: ahabashi@abrii.ac.ir

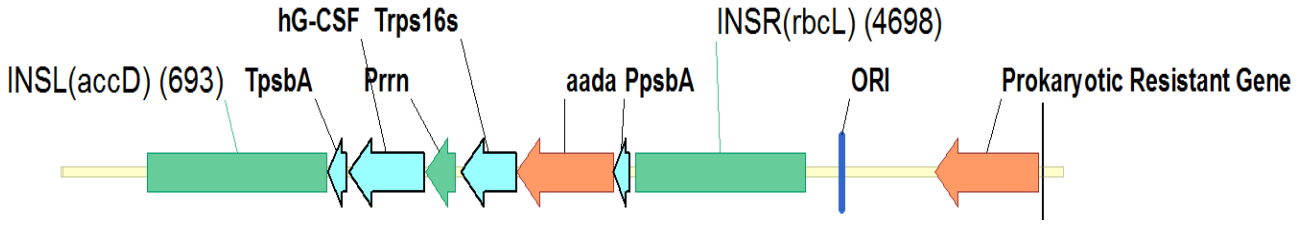

Fig. 1. pCL-hG-CSF chloroplast expression vector. *GUS* gene was replaced with *hG-CSF* gene.

acceptance, which make the plantspotentially one of the best factories for recombinant protein production [15]. On the other hand, the low level expression of produced proteins in plants prevents to achieve a chance for market competition. For overcoming nuclear overexpression limitations, which is most due to cosuppression phenomena in plant, the chloroplast platforms have been selected and developed since 2000. The higher level of expression was attained after developing chloroplast engineering techniques. The human growth hormone was expressed in tobacco chloroplasts more than 7% of total soluble protein [16]. In 2009, the highest recombinant protein accumulation in chloroplast platform was achieved for proteinaceous, more than 70% of the total soluble protein [17]. This issue means that this system with a high amount of recombinant protein expression can compete easily with *E. coli* and available eukaryotic systems.

Here, we developed the platform system based on the lettuce chloroplasts. We set up our expression platform with β-glucuronidase (GUS) reporter gene. Then, the *hG-CSF* gene was cloned into plant chloroplast expression chloroplast vector. This construct was amplified in *E. coli* and used for transformation of lettuce plastomes. Transgene integration and expression in plastomes were confirmed by PCR and Western immunoblotting.

MATERIALS AND METHODS

Primers. In this study, two sets of primers were designed: Syngcsf-F, TTTTTACAACAATTAC CAAC; Syngcsf-R, TCATGGTTGTGCAAGATGT and Ftransplastomic, ATCACATCTTCAATCATTC; Rtrasplastomic, TAGAAATTCTTTAGTAGCG. Primers Syngcsf-F and Syngcsf-R were used for confirmation of recombinant *E. coli* strains by colony PCR and primary approval of gene transformation. The two other primers (Ftransplastomic and Rtrasplastomic) were used for verification of correct insertion of transgene into chloroplast genome.

Vectors. Two vectors were used in this study: pMA cloning vector containing *hG-CSF* gene and pCL vector containing *GUS* gene. The two genes were located between *Hind*III and *Sal*I restriction sites. Both vectors were cut with the same restriction enzymes and *hG-CSF* was inserted into pCL vector instead of *GUS* gene. The pCL vector contains Prrn16S promoter, which is a strong ribosomal promoter. Also, this vector includes 3' untranslated region of *psba* (photosynthetic reaction center protein) gene (TpsbA), which is one of the strongest transcription terminators in chloroplast genome. These fragments are harbored by flanking regions (accD and rbcL) for specific integration into chloroplast genome by homologous recombination. The final constructed vector was named pCL-*hG-CSF* (Fig. 1).

Construction of chloroplast transformation vector pCL-hG-CSF. The pCL containing *GUS* gene and pMA were transferred to *E. coli* and plasmid extraction was carried out using plasmid extraction kit (Fermentas, Germany). Both plasmids were digested by *Hind*III and *Sal*I and pCL vector and *hG-CSF* fragments were purified from agarose gel. Subsequently, ligation reaction was performed between pCL and hG-CSF fragments at 16ºC for 4 h. Afterward, recombinant plasmids were transferred to *E. coli*. *hG-CSF* fragments were replaced in GUS region of pCL under the control of Prrn16S promoter and the TpsbA terminator by ligation process. The recombinant colonies were selected on screening media and confirmed by colony PCR and digestion. Finally, the selected plasmid was applied for sequencing.

Preparation of gold particles. Gold particles (60 mg) in 1 micron in diameter were added to 1 ml absolute ethanol in a 1.5-ml Eppendorf tube and vortexed for 1 min. Microtubes were spun in a table microcentrifuge for 8 seconds and the ethanol was decanted. These steps repeated two times and then 1 ml distilled water was added and vortexed for 10 seconds. The microtubes were spun for 1 min and supernatant was decanted. This step was also repeated two times. Next, 1 ml distilled water was added to a microtube

and vortexed to make a homogenous gold particle suspension. The suspension was distributed to 1.5-ml microtubes in a manner that each microtube included 60-µl gold particle. Then, the microtubes were kept at 4°C.

Gene transformation of gold particles coated pCL-GUS and pCL-hG-CSF to lettuce chloroplast genome by gene gun. A biolistic particle delivery system (Bio-Rad, USA) was used for transformation experiments. The constant parameters were a 10-mm distance between macrocarrier and stopping screen, chamber vacuum pressure (914 mbar, 28 in Hg) and size of gold particles (1 micron). The distance of explants from stopping screen was 9 cm. The pressure of rupture disk was 7,584 kPa (1,100 psi).

Regeneration of transplastomic plants. Lettuce cotyledons were used as explant for transformation. After transformation, the explants were transferred into selected media, containing 6-benzylaminopurine (0.2 mg/L), 1-naphthaleneacetic acid (0.05 mg/L) and streptomycine (40 mg/L). The Petri dishes were placed in a growth chamber under 16:8 h (light/dark) photoperiod. For plant regeneration, callus subculturing (20 days for each subculture) was carried out for several times. The transplastomic plants were transferred to 1/2 Murashige and Skoog medium without any hormone, just streptomycine (25 mg/L).

PCR analysis of transplastomic plants. Total DNA of transplastomic plants was purified using cetyl trimethylammonium bromide CTAB method [18]. Specific PCR amplification for *hG-CSF* gene was performed using specific primers and transplastomic plants containing *hG-CSF* gene were detected. Syngcsf-F and Syngcsf-R primers were used for verification of recombinant construct and primary approval of plant gene transformation. The two other primers including Ftransplastomic and Rtrasplastomic were used for approval of correct integration of *hG-CSF* into insertion sites of rbcL and accD in chloroplast genome.

GUS assay of embryogenic calli containing GUS gene. Histochemical staining of lettuce was carried out using Jefferson and Kavanagh's protocol [19] with some modifications performed after gene gun transformation. The transformed androgenic calli were immersed in a phosphate buffer solution, containing 0.5 M NaH_2P0_4, 0.1% Triton X-100, 10 mM EDTA, 2 mM X-gluc (5-bromo-4-cloro-3 indolyl-β-D-glucuronide) and 0.78 µl 2-mercaptoethanol. A color of the solution was observed after 12 hours of incubation at 35°C.

Immunoblot assay. Leaf samples (300 mg) from transplastomics and control plants were grounded to fine powder and added to 300 µl extraction buffer consisting of 100 mM Tris-HCl and 50 mM 2-mercapthoethanol. Protein concentration was estimated by Bradford dye binding. Soluble proteins (50 µg) from each sample were loaded in a 12% SDS-polyacrylamide gel. After gel electrophoresis, the proteins were blotted to a polyvinylidene fluoride membrane (Bio-Rad, USA) by a wet transblotting apparatus (Bio-Rad, USA) based on manufacturer's instructions. The blotted membrane was incubated in TBST containing 5% non-fat dried milk at room temperature for 1.5 h. The membrane was incubated with rabbit anti-granulocyte CSF polyclonal antibody at a dilution of 1:5,000 at 4°C for 6 h. Subsequently, the membrane was washed three times with TBST for 15 min and then the membrane was incubated with peroxidase-labeled secondary antibodies against rabbit (1:7,000; Sigma, USA) at room temperature for 1 h. After washings three times in TBST, the bound antibodies were visualized by ECL® system (Sigma-Aldhrich, USA). Finally, the film was scanned with a densitometer (GS-800, Bio-Rad, USA).

RESULTS

Cloning the hG-CSF in chloroplast expression vector. The pCL chloroplast expression vector containing *GUS* gene was digested by *Hind*III and *Sal*I and showed two bands on agarose gel including *GUS* gene (1,800 bp) and pCL vector without *GUS* gene (~5 kb). The digested vector was extracted from agarose gel by gel purification kit (Fermentase, Germany). pMA vectors were digested by *Hind*III and *Sal*I in both sides of *hG-CSF* gene and a 700-bp *hG-CSF* fragment was purified by gel extraction kit (Fermentase, Germany). Both purified fragments (digested vector and insert) were checked on agarose gel and ligated by rapid ligation kit (Fermentase, Germany) at 16°C overnight. Then, the results were transformed to *E. coli*. The *hG-CSF* fragment which was harbored by flanking regions (accD and rbcL), was replaced in GUS region of pCL under the control of prrn16S and the psbA terminator (TpsbA). The recombinant colonies were selected on screening media and were studied by colony PCR and digestion of extracted recombinant plasmid by *Hind*III and *Sal*I. Finally, the selected plasmid was applied for sequencing and the sequencing results confirmed the cloning of hG-CSF in the plant chloroplast expression vector, pCL-93-69.

Regeneration of transplastomic plants. Lettuce cotyledons were exploited for bombardment. After transformation, cotyledons were transferred into

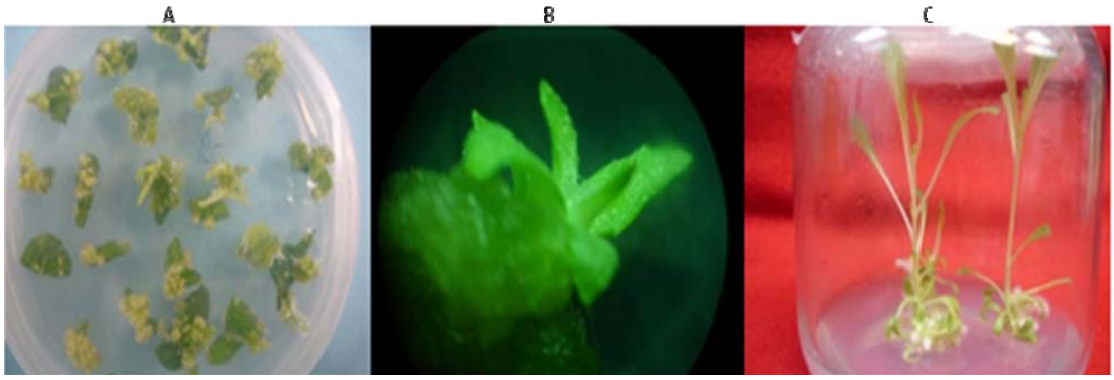

Fig. 2. Different regeneration stages of the transplastomic lettuce plants. **(A)** Explants on regeneration medium containing 6-benzylaminopurine (0.2 mg/L), 1-naphthaleneacetic acid (0.05 mg/L) and streptomycine (40 mg/L). **(B)** Four weeks tissues showing regeneration of adventitious shoot. **(C)** Shoot elongation achieved by transferring the clusters of adventitious shoot buds to medium (1/2 Murashige and Skoog media containing 25 mg/ml streptomycine).

Murashige and Skoog medium containing 6-benzyl-aminopurine (0.2 mg/L), 1-naphthaleneacetic acid (0.05 mg/L) and streptomycine (40 mg/L). The regenerated plants were maintained in a growth chamber at a photoperiod of 16 hours light and 8 hours darkness for one month. After subculturing, plantlet regeneration was appeared on the surface of calluses. The putative transplastomic plants were transported to 1/2 Murashige and Skoog medium without any hormone, just streptomycine for root induction (Fig. 2).

GUS assay of transplastomic plant containing GUS. After one month of lettuce embryo regeneration from calluses, the plantlets revealed GUS activity (Fig. 3). In this experiment, the GUS activity was detected in phosphate buffer (pH 7) without methanol in the reaction buffer. Gus staining data strongly confirmed the activity of the plastid specific promoter.

PCR analysis of transplastomic plants. Two pair of primers were used for PCR amplification from transplastomic plants genomic DNA to check presence of *hG-CSF* gene in targeted site. PCR results for gene specific primers were correct (700 bp) (Fig. 4A). Also, two special primers were used in a manner that forward primer was annealed to the end of *hG-CSF* gene sequence and backward primer was attached to complement region in chloroplast genome and amplified a 3-kb fragment (Fig. 4B). Amplification of 3-kb fragments showed that the insert was integrated in correct position in chloroplast genome.

Expression of hG-CSF gene in transgenic lettuce. The T_0 transgenic plants were analyzed for hG-CSF protein expression by Western blotting. Three out of four transgenic plants showed a protein band of the expected size (20 kDa), which was the result of protein reaction with hG-CSF antibody (Fig. 5). Untransformed control plant did not show any positive band for the hG-CSF protein.

DISCUSSION

Recombinant hG-CSF generates global sales of 5.6 billion USD (from June 2005 to June 2006) and its related market in Europe and United States of America has the big potential to sale close to 605

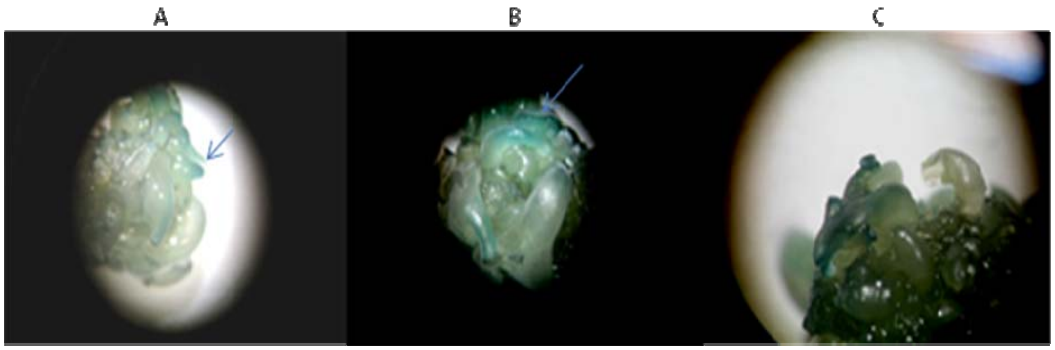

Fig. 3. GUS histochemical assay of transplastomic callus containing β-glucuronidase gene. Calli were stained by X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and incubated at 37°C overnight 3-4 weeks after bombardment. **(A and B)** Transplastomic calli and **(C)** negative control (non-transplastomic).

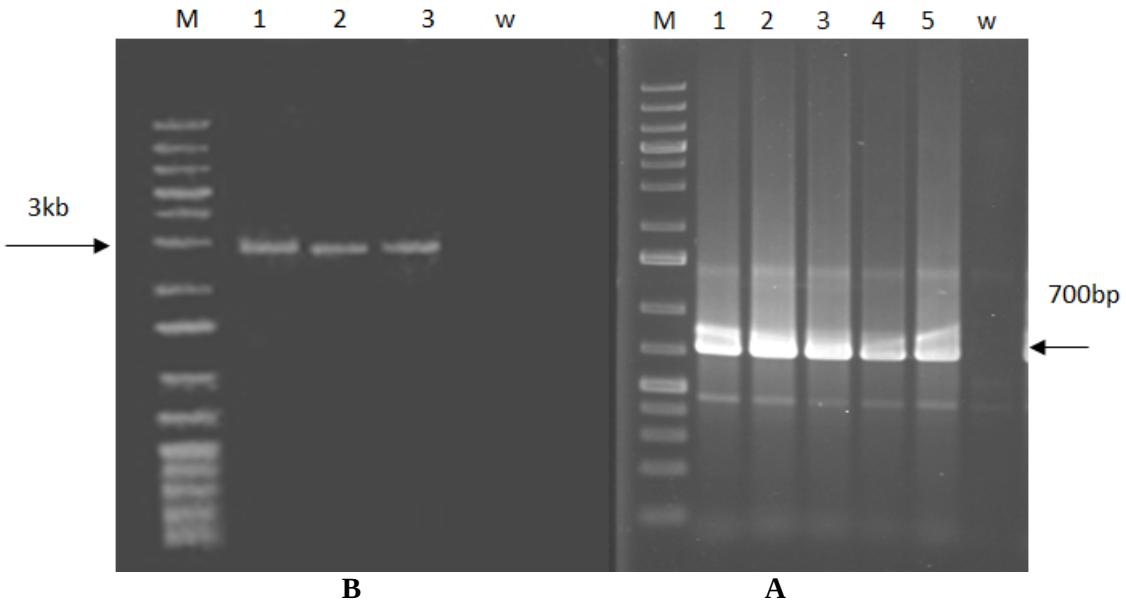

Fig. 4. Polymerase chain reaction analysis of transgenic genomes. **(A)** Specific PCR for transplastomic plants containing *hG-CSF* gene with primers for *hG-CSF* gene showed a 700-bp band. M, 1 kb plus DNA marker (Fermentas, Germany); lanes 1-5, transplastomic plants and w, Wild type plant. **(B)** PCR for transplastomic plants containing *hG-CSF* gene with forward primer for end of *hG-CSF* gene and backward primer for complement region in chloroplast genome amplified a 3-kb fragment. M, 1 kb plus DNA marker (Fermentas, Germany); lanes 1-3, transsplastomic plants and w, Wild type plant.

million USD [7]. Therefore, production of this recombinant protein is a prominent issue in biopharmaceutical industry. Classical systems for production of recombinant proteins such as *E. coli* expression fermentation system, CHO cell etc. have limitations in different aspects, including expenses, safety and scalability. Therefore, the researchers are searching for safer and cheaper substitutes for production of recombinant proteins.

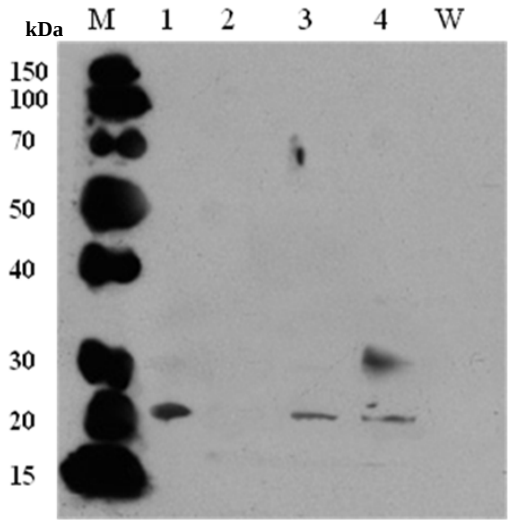

Fig. 5. Immunoblot analysis for detecting the expression of hG-CSF protein (20 kDa) in total soluble leaf protein of lettuce. M, protein marker (page ruler unstained broad range protein ladder, Thermo); Lanes 1-4, transgenic lines and w, wild type lettuce as negative control.

Transplastomic technologies provide a huge potential for recombinant protein production in plants, including the production of therapeutic substances, edible vaccines and antibodies.

When comparing chloroplast platforms with prokaryotic system, we can find some special advantages, such as elimination of fermentation process, relatively cheap investment, scalability, better folding and biosafety [20]. Large-scale production is a big advantage for using transplastomic plants in the recombinant industry. There is no need for expert employee to operate some complicated facilities such as bioreactors in the case of transplastomic plants. Therefore, investment and operating expenses are getting lower than other expression systems [21]. On the other hand, the low level expression in transgenic plants prevents to achieve a chance for market competition. The highest level expression reported is more than 70% of total soluble protein which is achieved in transplastomic strategy [17]. This high amount of recombinant protein expression can easily compete with other expression platform including *E. coli*. Chloroplast platform has also other advantages, such as no gene escape (due to lack of transgene in pollen), protein stability and no positional effect, correct folding of human proteins, no gene silencing, feasibility of marker excision and polycistronic expression.

In this study, we used pCL chloroplast expression vector. This vector had rbcL and accD flanking regions

for inserting the gene in the suitable place into the chloroplast genome. These regions are widely used in chloroplast expression vectors and their efficiency for homologous recombination has been already proved. The Prrn promoter and TpsbA terminator were used to increase hG-CSF expression in transcription level. All of these elements have been used in previous chloroplast researches [20] and effectively increased expression in transcription level.

On the other hand, the transplastomic regeneration rate was increased because of high level expression of *aada* (streptomycine resistant gene). This gene was cloned between psbA promoter and Trps16 terminator. These two elements play a key role in overexpression of *aada* gene through transcription enhancement and consequently, they cause transplastomic lines to be resistant to high dose of antibiotic concentration. The repeating of subculturing and regenerating calluses and plantlets helped to homoplasmy of chloroplast genome which means all of the chloroplast genomes contain gene of interest as well as selectable gene. This issue avoids the emergence of non-transformed plastomes which tend to escaped non-transplastomic lines.

The nuclear genome expression of *hG-CSF* gene has been already reported by our research team [22]. Here, we successfully examined the expression of *hG-CSF* gene in lettuce chloroplast genome as a first report. hG-CSF should serve as valuable recombinant biopharmaceutical for research and treatment of the human blood cancer. Also, this optimized expression platform can be considered as a suitable system for other recombinant proteins.

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