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آموزش مهارت های کاربردی در تدوین و چاپ مقاله
Human Granulocyte Colony-Stimulating Factor (hG-CSF) Expression in Plastids of Lactuca sativa

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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

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acceptance, which make the plant potentially 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 mostly 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 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, TTTTTACAACAATTACC AAC; Syngcsf-R, TCAATGTTGTGCAAAGATGT and Frtransplastomic, ATCACATCTTCAATCTTC; Rtrasplastomic, TAGAATTCTTTAGTACG. 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 (Frtransplastomic 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 HindIII and SalI 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 Prn16S 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 HindIII and SalI and pCL vector and hG-CSF fragments were purified from agarose gel. Subsequently, ligation reaction was performed between pCL and hG-CSF fragments at 16°C 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 Prn16S 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 Rtransplastomic 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 NaH₂PO₄, 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-mercaptoethanol. 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-Aldrich, 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 HindIII and SalI 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 HindIII and SalI 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 prn16S 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 HindIII and SalI. 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

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Murashige and Skoog medium containing 6-benzylaminopurine (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 T₀ 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 billion USD.
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.

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 (streptomycin 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 homoplasy 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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