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Original paper

Expression of human interferon gamma in tobacco chloroplasts

SHAHLA RAZMI[1], MOKHTAR JALALI JAVARAN[1*], ABDOLREZA BAGHERI[2], HOSSEIN HONARI[3], MOJGAN SOLEIMANI ZADEH[2]

[1]Biotechnology and Plant Breeding Department, Agriculture Faculty, Tarbiat Modares University, Tehran, Iran
[2]Biotechnology and Plant Breeding Department, Ferdowsi University of Mashhad, Mashhad, Iran
[3]Biology Department, Science Faculty, Imam Hossein University, Tehran, Iran

Abstract Chloroplast genetic engineering enhances capacity of plants as a bioreactor for the production of recombinant proteins through increasing transgenes expression level and protein accumulation. In this feasibility study, the potential of high-yield expression of interferon gamma (IFNg), a valuable therapeutic human protein, is explored for tobacco chloroplasts. The interferon gamma gene (ifnG) and aminoglycoside 3'-adenylyltransferase (aadA) (as a selectable marker gene) were introduced into the inverted repeat (IR) of tobacco plastome as a dicistronic operon using a biolistic-mediated transformation method. The presence and expression of ifnG in the regenerated plants were confirmed in DNA, RNA, and protein levels by polymerase chain reaction (PCR), reverse transcription-polymerase chain reaction (RT-PCR), and Western blot analysis, respectively. Furthermore, quantification of expressed IFNg by Enzyme-linked immunosorbent assay (ELISA) showed that the level of expression in transplastomic plants was approximately 0.42% of the total soluble proteins (TSPs), which is much higher than its expression in nuclear transformed plants. Southern blot analysis was used to confirm homoplasmy in the T0 progeny. Overall, the results showed that the plastid transformation can be employed as an effective tool for large-scale production of pharmaceutical proteins. Moreover, dicistronic expression of ifnG and aadA genes in tobacco chloroplasts simplifies gene manipulation.

Keywords Dicistronic operon, Pharmaceutical proteins, Plastid transformation/

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*Corresponding author: SHAHLA RAZMI, Biotechnology and plant breeding department, Agriculture faculty, Tarbiat Modares University, P.O. Box: 1411713116, Tehran, Iran, Tel: +98-21-44580500
E-mail: jalali.mokhtar@gmail.com

Introduction

The possibility of performing plastid transformation in higher plants increases the appeal of this expression system in the production of pharmaceutical proteins at biological manufacturing sites (P. MALIGA [1]). Plastid transformation has many advantages over nuclear transformation. Some of the advantages of plastid transformation are the ability to accumulate large amounts of foreign proteins due to the polyploidy of the plastid genome, the lack of position effect and gene silencing due to site-specific integration into the plastome, the absence of transgene dissemination via pollen transmission due to maternal inheritance, and the capacity to express several genes simultaneously due to the operonic model in plastids (D. VERMA & al. [2]). Furthermore, applying appropriate post-translational modifications and producing functionally active chloroplast-derived therapeutic proteins have already paved the way for the production of cost-effective plant-based therapeutic proteins (H. DANIELL & al. [3]). Several proteins with mammalian origin have been expressed successfully in tobacco chloroplasts such as human somatotropin up to 7% total soluble proteins (TSPs) (J.M. STAUB & al. [4]), human serum albumin up to 11.1% TP (F.S. MILLáN & al. [5]), interferon alpha2b up to 20% (P.A. ARLEN & al. [6]), insulin up to 32% TSPs (H. DANIELL & al. [7]), etc. Here we focused on interferon gamma protein (IFNg), a hunam theraputic glycoprotein which is secreted by activated T lymphocytes and natural killer cells (M.A. FARRAR & al. [8]). The biologically active form of IFNg is a homodimer (T. SARENEVA & al. [9]). IFNg has many valuable clinical applications in viral and bacterial infections as well as in anti-parasitic therapy (M. FRESE & al. [10], C. NAUCIEL & al. [11], J. ASSREUY & al. [12]). So far, the only therapeutically used recombinant IFNg, is the one produced in *Escherichia coli* (Actimmune) (R.B. FREEDMAN & al. [13]) but it is still very expensive especially in developing countries. To date, several groups have tried to develop a less costly system by employing plants as hosts and producing transgenic plants that have interferon gamma gene (*ifnG*) in their nuclear genome such as rice (T.L. CHEN & al. [14]), tomato (N. EBRAHIMI & al. [15]), tobacco (S. LEELAVATHI & al. [16], Y. WU & al. [17], M. MORI & al. [18]) and rapeseed (K. BAGHERI & al. [19]), but the expression level of *ifnG* achieved through this method is still very low. Chloroplast transformation strategy has been also used for the production of large amount of IFNg, but the yield of production is not satisfactory too (S. LEELAVATHI & al. [16]). Therefore, we decided to increse the efficiency of chlorplast transformation by integration of *ifnG* in a proper insertion site in the inveretd repeat (IR) region of tobacco plastome. Furthemore we were going to verify the feasibilty of dicistronic expression of *ifnG* and a marker gene, aminoglycosid 3'- adenyltransferase gene (*aadA*) which confers to spectinomycin and streptomycin resistance, in an operon in order to facilitate gene manipulation (S.W. JEONG & al. [20]) .

Material and methods

Cloning of ifnG in the plastid transformation vector

For plastid transformation the pKCZ vector was used (courtesy of Dr Hashemi, National Institute of Genetic engineering and Biotechnology) that is designated for integration of foreign genes between *trnR*-ACG and *trnN*-GUU inside the tobacco plastome by double recombinations between similar sequences (Z. ZOU [21]). We cloned *ifnG* to this vector between the promoter of the *rRNA* gene (Prrn) and *aadA* using the *Nco*I restriction enzyme to be expressed dicistronically with *aadA*. At first, *ifnG* was amplified using the following primers: forward primer (F1), 5'catgccatggaacatcatcatcatcatcatcaggacccatatgtaaaag; reverse primer (R), 5'catgccatggatcctcctccttcattactgggat gctcttcgac. The F1 primer contained a His-tag sequence and a restriction site for *Nco*I, while the R primer contained a ribosome-binding site (RBS) sequence and a restriction site for *Nco*I. His-tag was added for use during IFNg purification later. The *Nco*I restriction site in the original pKCZ vector is located after the RBS for *aadA* and therefore RBS will be separated from the *aadA* translation start site by insertion of *ifnG*. That is why we have added a new RBS for *aadA* by including it in our reveres primer sequence. Both amplified *ifnG* and the pKCZ vector were digested with the *Nco*I enzyme prior to ligation and transformation. Successful insertion of *ifnG* was confirmed by colony polymerase chain reaction (PCR) using F1 and R primers and sequencing of the product. Since we used just one restriction enzyme for cloning, we had to check the direction of the gene inside the vector. For this purpose, we did a PCR with following primers; forward primer (F2): 5´ggctagcggcaattcgccgtc gttcaatgagaatthat (against 5'-end of the Prrn sequence) and R primer. Providing the *ifnG* had been successfully inserted in the same direction with the *aadA* gene, an approximately PCR product of 500 bp is expected; otherwise a 100 bp fragment will be amplified.

Plastid transformation

Tobacco seeds (*Nicotiana tabacum* cv. Zanthi) were aseptic and grown on Murashige and Skoog (MS) medium containing 30 g /L sucrose under a 24-hour cycle including 16 hours of light and 8 hours of darkness. After several weeks, the dark-green leaves of these plants (whose lengths were more than 5 cm) were selected for bombardment. These leaves were pre-treated for 24 hours before shooting. *IfnG*::pKCZ was then coated onto 0.6 µm gold particles and coated particles were used for the bombardment of leaves using a biolistic gun (Bio-rad, Munich, Germany). After bombardment, the leaves were kept in darkness for 48 hours, then cut into small pieces and transferred to the RMPO medium with 500 mg/L spectinomycin (Z. SVAB & al. [22]). After 4 to 6 weeks, the first plants were regenerated. These plants were transferred to an MS medium for rooting. To make sure these plants did not result from mutations; their leaves

were cut into small pieces and transferred on RMOP medium containing 500 mg/L streptomycin.

PCR analysis

Total DNA was extracted from the leaves of plants using cetyl trimethyl ammonium bromide (CTAB) method (M. MURRAY & al. [23]). To confirm the presence of the *ifnG* gene in these plants, a PCR assay was performed using the F1 and R primers. For further confirmation of gene insertion at the directed site into the plastome, an additional PCR assay using the following primers was performed: forward primer (F3), 5'gcatctaagtagtaagcccaccccaagatg complementary to a region close to the *trnR* (between *rrn5* and *trnR*) (R.E. SORIA-GUERRA & al. [24]) and R primer. The distance between these primers is about 2 kb (Figure 1).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction was performed using RNX-plus™ (Sinagen, Tehran, Iran). The pellet was dissolved in suitable amount of diethylpyrocarbonate (DEPC) water followed by treatment with *DNase*I enzyme (Takara). 2 µg of extracted RNA and reverse transcription enzyme (RT-PCR kit, Fermentas) with a reverse primer of *ifnG* at 37ºC were used for cDNA synthesis. RT-PCR was performed using specific primers of *ifnG*. To show that the extracted RNA was DNA-free, an additional PCR was performed using only extracted RNA as template.

Protein extraction and Western blot

Total soluble proteins (TSPs) from wild-type and transplastomic plants were extracted in extraction buffer (0.7 M sucrose, 0.5 M Tris HCl, 50 mM EDTA, 0.1 M KCl and 2% β-Mercaptoethanol). After a short time vortex, phenol was added and centrifuged for 10 minutes at 12470 xg. Proteins were precipitated by adding ammonium acetate dissolved in methanol, followed by incubation overnight at –20ºC and centrifugation for 5 minutes at 12470 xg. The precipitated pellets were dissolved in sodium dodecyl sulfate (SDS) 1%. For western blot analysis, the proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were blotted onto a nitrocellulose membrane (Bio-Rad) with a semi-dry transfer cell (Trans-blot SD cell, Bio-Rad; USA). The membrane was incubated overnight at 4ºC using blocking buffer (1x phosphate buffered saline (PBS), 0.05% Tween 20 and 5% w/v non-fat dry milk). The membrane was washed 3 times with washing buffer (1x PBS, 0.1% Tween 20) and incubated with the primary antibody (rabbit-anti IFNg polyclonal antibody, dilution 1:200, Santa Cruz) for 1 hour at 37ºC. After being washed 3 times, it was incubated with secondary antibody (anti-goat IgG horseradish peroxidase conjugated, 1:500, Santa Cruz) for another hour at 37ºC. Final washing was done to remove secondary antibody and the membrane was incubated in substrate (diaminobenzidin (DAB), H_2O_2 and PBS) for 15 to 20 minutes. The reaction was stopped with distilled water.

Enzyme-linked immunosorbent assay (ELISA)

A coating buffer was mixed with 40 µl of TSPs from each of the transplastomic and wild-type plants, which were then separately loaded into a 96-well microtiter plate. Microtiter plate was incubated overnight at 4ºC. After blocking by 1% bovine serum albumin (BSA), the primary antibody (dilution 1:200, 100 µl per well) and secondary antibody (dilution 1:1000, 100 µl per well) was added to the plate respectively, and incubated at 37°C for 1 hour. Washing was performed at the end of each stage by PBS-T. Following incubation with 100 µl substrate solution (1% 3, 3', 5, 5'-tetramethylbenzidin (TMB), 200 mM citrate buffer, and 0.01% H2O2) per well for 20 minutes in a dark room, reactions were stopped with a 2 N HCl solution. The absorbance was measured for each well at 450 nm in three replicates using a microplate reader (Anthos 2020). The average of three measurements for each transplastomic and wild-type plants were compared using the *t*-test at the 5% significance level. The protein quantification of transplastomic plants was then performed by applying a standard curve generated by measuring the absorbance of the commercial IFNg (Imukin) with 0.5, 1.5, 2.5, 5, 10, and 20 ng quantities.

Southern blot analysis

Southern blot analysis was performed on four independent adult transplastomic plants and one wild-type plant according to the DIG- DNA labeling kit instructions (Roche, Germany) after digesting extracted DNAs by *Hind*III. A 369 bp-labeled probe was prepared complementary to the *trnR* region existing in the tobacco plastome (Figure 1) and was used for detection according to the manufacturer's instructions.

Results and Discussion

Cloning of ifnG in a chloroplast vector

IfnG was inserted into the plastid transformation vector pKCZ between the Prrn and RBS of *aadA*. The *16s rRNA* promoter (Prrn) is a constitutive promoter in chloroplasts and prokaryotes that drives in this construct both *ifnG* and *aadA* genes. The selectable marker gene, *aadA* is used for selection of resistant transformants in the regeneration medium. The RBS is a 26-bp sequence comprising a canonical Shine-Dalgarno (SD) sequence (GGAGG) which is employed to initiate translation of *ifnG*. Furthermore an additional RBS (containing only the canonical sequence) was included for *aadA* translation separately. Given these conditions, we prepared a dicistronic construct for *ifnG* and *aadA* in the pKCZ (Figure 1).

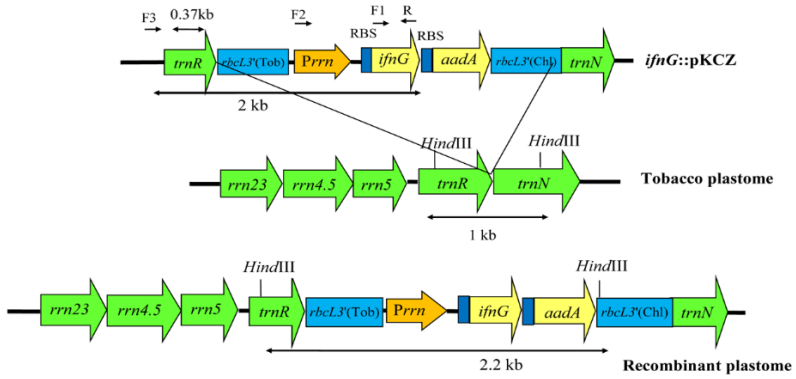

Fig. 1 Plastid transformation vector pKCZ harboring *ifnG* and *aadA* gene under the control of one promoter (Prrn) and terminator (rbcL3'chl) but separate RBSs. Integration occurs at the *trnR/trnN* insertion site into the tobacco plastome. F1, F2, F3 and R show primers used for PCR. Probe 0.37 kb is marked upon *trnR* in the vector used for southern blot analysis.

Regeneration of tolerant plants on the selective medium

The constructed vector was used to transfer the *ifnG* to the tobacco chloroplasts by micro projectile bombardment. After 6 to 7 weeks, several regenerated plants were seen on the selective medium containing 500 mg/L spectinomycin (Figure 2a), while wild-type plant leaves were not able to regenerate and became yellow (Figure 2b). As resistance to spectinomycin possibly occurs due to random mutations, an extra antibiotic resistance is tested (R. BOCK [25]). Therefore, we tested the regeneration of plants on the selective medium containing 500 mg/L streptomycin. Results showed that most of the transplastomic plants were resistant to streptomycin (Figure 2c), while non-transformed plant leaves became yellow (Figure 2d). The regenerated plants were transferred to MS medium containing spectinomycin to generate roots (Figure 2e). Subsequently, plants with resistance to both spectinomycin and streptomycin were subjected to four rounds of regeneration to achieve homoplasmy. Finally these plants were transferred to Perlit (Figure 2f) and, after adaptation in greenhouse conditions, they were transferred to pots (Figure 2g) to be grown and produce seeds.

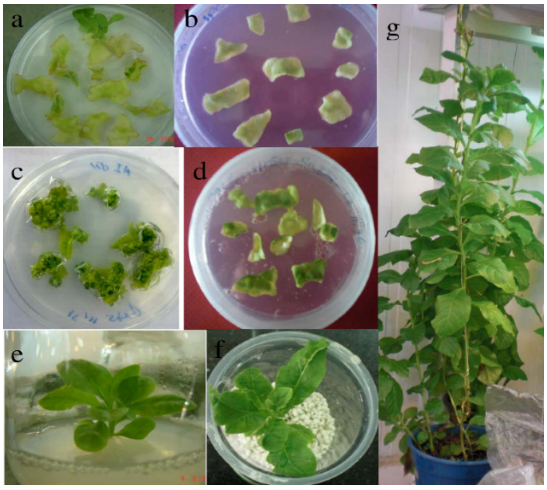

Fig. 2 a Direct regeneration of bombarded leaves on media containing 500 mg/L spectinomycin; **b** Wild-type of tobacco on media containing 500 mg/L spectinomycin; **c** Regeneration of transformed plants on the media containing 500 mg/L streptomycin; **d** Wild-type of tobacco on media containing 500 mg/L streptomycin **e** Root formation in the MS media containing 500mg/L spectinomycin; **f** Resistant plants in Perlit; and **g** Adult plant with capsules in the pot.

Confirming the integration of ifnG in the plastome by PCR

To confirm the presence of *ifnG* in the regenerated resistant plants, genomic DNA was isolated from their leaves and amplifications were performed using F1 and R primers (Figure 3). Also, insertion of *ifnG* gene into the expected right site in the plastome (between *trnR* and *trnN)* was verified by a PCR using F3 and R primers (Figure 1), yielding an expected 2 kb product in transplastomic plants (Figure 4).

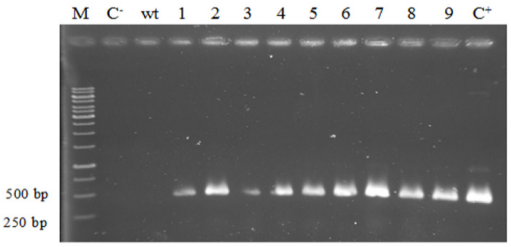

Fig. 3 PCR analysis using specific primers (F1 and R) for *ifnG*. A product of approximately 500 bp was found in only transplastomic plants. M: 1kb ladder; C^-: negative control; wt: wild-type plant; number 1 to 9: transplastomic plants; C^+: positive control (*ifnG*::pKCZ was used as template).

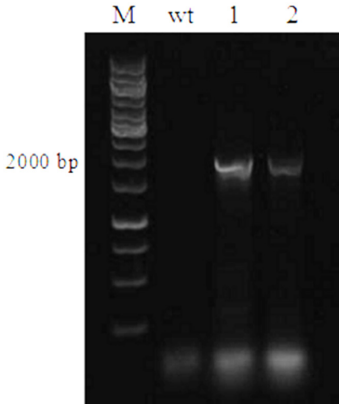

Fig. 4 PCR analysis using the F3 and R primers to confirm *ifn*G insertion into the right site of the plastome: a 2-kb band was seen in only transplastomic plants (number 1 and 2) but not seen in wild-type plant (wt).

RNA analysis

Total RNA was extracted and all genomic and plastid DNA was removed by *DNase*I enzyme. Next cDNA was synthesized from the total RNA of both transplastomic and wild-type plants. Then RT-PCR was performed for assaying of the *ifnG* transcription. Our results showed that *ifnG* is transcribed in transplastomic plants successfully (Figure 5).

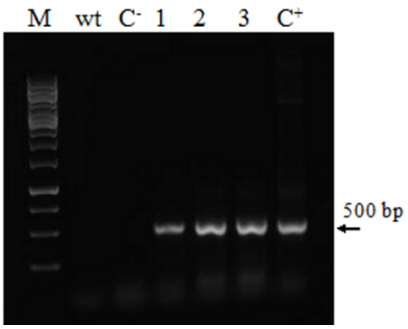

Fig. 5 confirmation of *ifnG* transcription by RT-PCR analysis. A specific 500-bp band was detected in transplastomic plants by using F1 and R primer of *ifnG*. M: 1kb ladder; wt: wild-type plant; C^-: extracted RNA used as template for PCR; number 1 to 3: synthesized cDNA used as template; C^+: positive control, *ifnG*::pKCZ used as template

Western blot analysis and ELISA

In the Western blot assay with Imukin (IFNγ commercial form) used as a positive control, two bands were shown at around 15 kDa and 30 kDa that are slightly lower than the predicted molecular weights of non-glycosylated monomerci and dimeric forms of IFNg (16 kDa and 32 kDa), respectively (T. SARENEVA & al. [9]); both the monomeric and dimeric forms of expressed IFNg in transplastomic plants were detected (Figure 6). Two different molecular weight forms were seen in the monomeric area (around 14 kDa and 15 kDa), likely corresponding to the truncated C terminus and natural IFNg, respectively (K. TAKEHARA & al. [26]). The carboxy terminal end of IFNg is susceptible to post-translational proteolysis due to the 9-16 residues being disordered or highly flexible (S.L. LAUREN & al. [27], R. MIRONOVA & al. [28], T. SARENEVA & al. [29]). It has been demonstrated that the truncated IFNg has similar biologic activity (K. TAKEHARA & al. [26]). Subsequently, two bands (around at 28 kDa and 32 kDa) were found in the dimeric area of IFNg: a 28 kDa protein which is probably the dimer of the 14 kDa protein, and a 32 kDa protein that is higher than that of Imukin due to the two accompanied His-tag linked to the two chains of IFNg. Also ELISA results showed that the average absorbance measurements for the transplastomic plants were significantly different from the wild-type at a 5% significance level (Figure 7) and the expression level of IFNg in these plants was approximately 0.42% of TSPs.

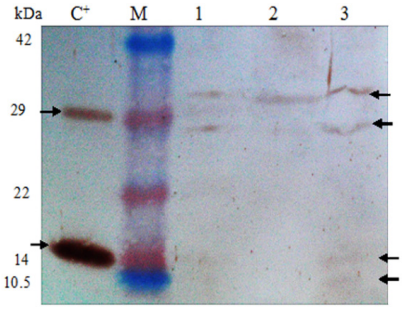

Fig. 6 Western blot analysis of transplastomic plants. C^+: Imukin (IFNg commercial form) has demonstrated both monomeric and dimeric forms; M: chromatin pre-stained protein ladder (Vivantis, bands in the range of ~10.5 – ~175 kDa); numbers 1 to 3 transplastomic plants. The arrow shows IFNg in transplastomic plants in monomeric and dimeric forms

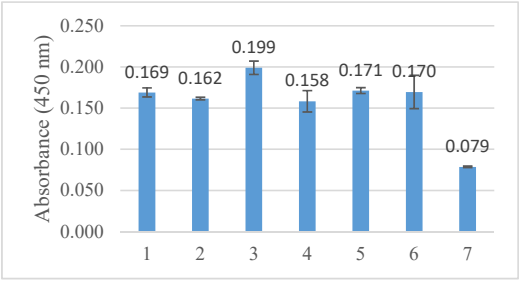

Fig. 7 ELISA results of transplastomic plants at OD 450 nm. 40 µl of TSPs from transplastomic plants were used to detect the presence of IFNg. Values are the means of three replicates along with standard deviations. Bar 1 to 6 represent the transplastomic plants, while bar 7 represents the wild- type plant

Southern blot analysis

Southern blot analysis was carried out to distinguish homoplastomic and heteroplastomic plants. According to the organization of the recombinant and wild-type plastome presented in Figure 1, 2.2 kb and 1 kb fragments were expected in putative transplastomic and non-transformed plants, respectively. Only the 2.2 kb fragment in transplastomic plants demonstrated the absence of any wild-type DNA in their chloroplasts, and thereby confirming homoplasmy (Figure 8). Although all of the four plants undergone four rounds of regeneration on the selective medium, with integration of transgene confirmed by PCR amplification (Figure 8), in two transplastomic plants (first and second lanes) both the fragments of 2.2 kb and 1 kb demonstrated heteroplasmy. This finding can likely be attributed to the remaining few wild-type plastomes in their chloroplasts and their propagation during plant growth in contrast with the plantlet stage tested by PCR and the adult stage tested via Southern blot assay.

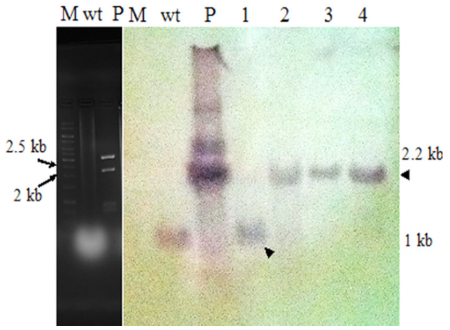

Fig. 8 Southern blot analysis of transplastomic tobacco plants. In this analysis, the 0.37 kb fragment of the *trnR* gene was used as a probe and detected a 1 kb fragment in wild-type plant and a 2.2 kb fragment in transplastomic plants after digestion with *Hind*III. M: 1kb ladder; wt: wild-type tobacco plant, P: digested *ifnG*::pKCZ with *Hind*III; numbers 1 to 4: transplastomic plants; numbers 1 and 2 showed both 1 and 2.2 kb fragments and are heteroplastomic plants while numbers 3 and 4 showed only the 2.2 kb fragment and are homoplastomic plants

In this report, efforts were made to increase the expression of IFNg in tobacco plants via plastid transformation. Previous research has documented independent IFNg expression in tobacco chloroplasts with low production yields up to 0.1% of TSPs (S. LEELAVATHI & al. [16]). Building on this, we attempted to improve the IFNg expression level by inserting the transgene into the plastome between two *tRNA* genes (*trnN-GUU* and *trnR-ACG*) in the IR region rather than in the large single copy (LSC) region. Gene insertion in the IR region has two advantages: the transgene integrated into this region is undoubtedly duplicated by copy correction in each plastome, which enhances gene transcription efficiency, and the *tRNA* gene transcripts surrounding the target gene are thought to increase mRNA stability in this method (Z. ZOU [21], S. RUF & al. [30]). Additionally, we hoped that using RBS, including the SD sequence upstream of *ifnG*, could improve translation efficiency. In this study, the IFNg protein content measured by ELISA was 0.42% of TSPs. Although, this result was much higher than the amount obtained from the nuclear transformant plants (0.001% and 0.03% oTSPs) (S. LEELAVATHI & al. [16], Y. WU & al. [17]) and higher than the IFNg expression level in chloroplasts in LSC region (0.1% of TSPs), it was much lower than the protein content obtained in the chloroplast transformation study, in which IFNg produced as a GUS fusion that rose to 6% TSPs (S. LEELAVATHI & al. [16]). Although we provided suitable conditions for the proper

transcription and translation of *ifnG* (including a strong constitutive promoter, high copy of mRNAs, mRNA stability, and efficient translation), we were not successful in achieving high-yield production. It seems that the low accumulation of IFNg in the chloroplasts can be attributed to a lack of post-translational modification. Chloroplasts support some of post-translational modifications like folding, disulfide bound formation and proper lipid modification, but does not support glycosylation (H. DANIELL & al. [3]). The human IFNg contains two potential N-linked glycosylation sites (T. SARENEVA & al. [9]). However, the glycosylation is not required for its antiproliferative and antiviral activity, the glycosylated forms might be more stable and less susceptible to proteolytic cleavages (E. RINDERKNECHT & al. [31]). Therefore, the plastid-derived IFNg is unstable and therefore will not accumulate as much as other non-glycosylated proteins produced by chloroplast transformation method. Fussing the instable proteins with a stable protein like GUS (β-glucuronidase) or cholera toxin B (CTB) subunit (D. BOYHAN & al. [32]) has been recommended by some researchers to increase unstable proteins accumulation. However, purification of the proteins of interest from the fused proteins is laborious and expensive, and approximately 25% of the proteins would be lost during the purification process.

Furthermore, in this study we expressed IFNg using a dicistronic operon which is cost and time effective (H. DANIELL & al. [33], Y. ARAI & al. [34]). Polycistronic expression has been successfully shown in production of Poly(R)-3-hydroxybutyrate (Y. ARAI & al. [34]), the recombinant DPT (diphtheria, pertussis and tetanus) vaccine as a fusion protein in tobacco chloroplast (R.E. SORIA-GUERRA & al. [24]) and simultaneous expression of GFP (green fluorescent protein) with a selectable marker in tobacco chloroplast (S.W. JEONG & al. [20]). Our study was also one successful example for dicistornic expression in chloroplast. It is indicated that some of (di-) polycistornic mRNAs undergo intercistronic cleavages to form discrete transcripts which are competent for translation (O. DRECHSEL & al. [35]). Successful translation of the dicistornic mRNA comprising of two mRNAs (*ifnG* and *aadA)* in this study indicated that the separation of these two mRNAs by intercistronic cleavage is not necessary.

Southern blot analysis showed that the first and second transplastomic plants have high and low levels of wild-type plastome, respectively, even after four cycles of subculturing. In addition, the spontaneous loss of homoplastomic plants is likely a consequence of removing essential genes located between direct repeats of Prrn from tobacco plastomes via intramolecular recombination. Here, the plastid promoter Prrn that governs both the selectable marker (*aad*A) and the transgene (*ifn*G) in the transplastomic plants has been aligned at the same direction with the Prrn that promotes endogenous *rRNA* genes. Recombination between these two direct repeats leads to the loss of the DNA fragment located between the two repeats, including the vital *rrn16* and *rrn23* genes. Consequently, these transplastomic plants will lose their viability (Z. ZOU [21]). Nevertheless, some of the transplastomic plants survive, probably due to keeping wild-type plastome in their plastids. The repeated cycles of antibiotic selection could eliminate wild-type plastome in plastids; on the other hand, losing the essential genes in the transplastomes (and hence the transgene) adversely increases heteroplasmy. Finally, it seems that the level of wild-type plastome increases after removing the transplastomic plants form the selective medium.

Conclusion

In this study, we successfully used tobacco chloroplasts as a bioreactor for expression of IFNg. In general, employing chloroplasts is a suitable solution to accumulate large amount of foreign proteins due to the high polyploidy of their genomes, but using chloroplasts as an inexpensive platform to produce proteins with short half-life and extensive post-transcriptional processing, requires further investigations.

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References

1. P. MALIGA, Plastid transformation in higher plants. *Annu. Rev. Plant. Biol.*, 55: 289-313 (2004).
2. D. VERMA, H. DANIELL, Chloroplast vector systems for biotechnology applications. *Plant. Physiol.*, 145(4): 1129-1143 (2007).
3. H. DANIELL, N.D. SINGH, H. MASON, S.J. STREATFIELD, Plant-made vaccine antigens and biopharmaceuticals. *Trends. Plant. Sci.*, 14(12): 669-679 (2009).
4. J.M. STAUB, B. GARCIA, J. GRAVES, P.T. HAJDUKIEWICZ, P. HUNTER, N. NEHRA, V. PARADKAR, M. SCHLITTLER, J.A. CARROLL, L. SPATOLA, High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat. Biotechnol.*, 18(3): 333-338 (2000).
5. F.S. MILLáN, A. MINGO-CASTEL, M. MILLER, H. DANIELL, A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant. Biotechnol. J.*, 1(2): 71-79 (2003).
6. P.A. ARLEN, R. FALCONER, A. COLE, S. CHERUKUMILLI, A.M. COLE, K.K. OISHI, H. DANIELL, Field production and functional evaluation of chloroplast-derived interferon-alpha2b. *Plant. Biotechnol. J*, 5(4): 511-525 (2007).
7. H. DANIELL, G. RUIZ, B. DENES, L. SANDBERG, W. LANGRIDGE, Optimization of codon composition and regulatory elements for expression of human insulin

like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. *BMC Biotechnol.*, 9(1): 33-49 (2009).
8. M.A. FARRAR, R.D. SCHREIBER, The molecular cell biology of interferon-gamma and its receptor. *Annu. Rev. Immunol.*, 11(1): 571-611 (1993).
9. T. SARENEVA, J. PIRHONEN, K. CANTELL, I. JULKUNEN, N-glycosylation of human interferon-gamma: glycans at Asn-25 are critical for protease resistance. *Biochem. J.*, 308: 9-14 (1995).
10. M. FRESE, V. SCHWARZLE, K. BARTH, N. KRIEGER, V. LOHMANN, S. MIHM, O. HALLER, R. BARTENSCHLAGER, Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology.*, 35(3): 694-703 (2002).
11. C. NAUCIEL, F. ESPINASSE-MAES, Role of gamma interferon and tumor necrosis factor alpha in resistance to *Salmonella typhimurium* infection. *Infect. Immun.*, 60(2): 450-454 (1992).
12. J. ASSREUY, F.Q. CUNHA, M. EPPERLEIN, A. NORONHA-DUTRA, C.A. O'DONNELL, F.Y. LIEW, S. MONCADA, Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major. *Eur. J. Immunol.*, 24(3): 672-676 (1994).
13. R.B. FREEDMAN, N. JENKINS, D.C. JAMES, M.H. GOLDMAN, M. HOARE, R.W.A. OLIVER, B.N. GREEN, Posttranslational processing of recombinant human interferon-γ in animal expression systems. *Protein. Sci.*, 5(2): 331-340 (1996).
14. T.L. CHEN, Y.L. LIN, Y.L. LEE, N.S. YANG, M.T. CHAN, Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures. *Transgenic. Res.*, 13(5): 499-510 (2004).
15. N. EBRAHIMI, H.R. MEMARI, M.A. EBRAHIMI, M.R. ARDAKANI, Cloning, Transformation and expression of human gamma interferon gene in tomato (Lycopersicon Esculentum Mill.) *Biotechnol. Biotechnol. Equip.*, 26: 2926-2929 (2012).
16. S. LEELAVATHI, V.S. REDDY, Chloroplast expression of His-tagged GUS-fusions: a general strategy to over-produce and purify foreign proteins using transplastomic plants as bioreactors. *Mol. Breed.*, 11(1): 49-58 (2003).
17. Y. WU, D. ZHAO, L. SONG, W. XU, Heterologous expression of synthetic chicken IFN-γ in transgenic tobacco plants. *Biologia.*, 64(6): 1115-1122 (2009).
18. M. MORI, G.-H. ZHANG, M. KAIDO, T. OKUNO, I. FURUSAWA, Efficient production of human gamma interferon in tobacco protoplasts by genetically engineered brome mosaic virus RNAs. *J. Gen. Virol.*, 74(7): 1255-1260 (1993).
19. K. BAGHERI, M.J. JAVARAN, F. MAHBOUDI, A. MOEINI, A. ZEBARJADI, Expression of human interferon gamma in Brassica napus seeds. *Afr. J. Biotechnol.*, 9(32): 5066-5072 (2010).
20. S.W. JEONG, W.J. JEONG, J.W. WOO, D.W. CHOI, Y.I. PARK, J.R. LIU, Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for selection and tracking of plastid-transformed cells in tobacco. *Plant. Cell. Rep.*, 22(10), 747-751 (2004).
21. Z. ZOU, Analysis of cis-acting expression determinants of the tobacco psbA 5'UTR in vivo, Ludwig Maximilian University of Munich, Germany, 2002, pp. 59-112.
22. Z. SVAB, P. MALIGA, High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Nation. Acad. Sci.*, 90(3): 913-917 (1993).
23. M. MURRAY, W.F. THOMPSON, Rapid isolation of high molecular weight plant DNA. *Nucl. Acids. Res.*, 8(19): 4321-4326 (1980).
24. R.E. SORIA-GUERRA, A.G. ALPUCHE-SOLíS, S. ROSALES-MENDOZA, L. MORENO-FIERROS, E.M. BENDIK, L. MARTíNEZ-GONZáLEZ, S.S. KORBAN, Expression of a multi-epitope DPT fusion protein in transplastomic tobacco plants retains both antigenicity and immunogenicity of all three components of the functional oligomer. *Planta.*, 229(6): 1293-1302 (2009).
25. R. BOCK, Transgenic plastids in basic research and plant biotechnology. *J. Mol. Biol.*, 312(3): 425-438 (2001).
26. K. TAKEHARA, M. KAMIKAWA, N. OHNUKI, T. NAGATA, A. NAKANO, D. YAMAGUCHI, Y. YOKOMIZO, M. NAKAMURA, High level expression of C-terminal truncated recombinant chicken interferon-gamma in baculovirus vector system. *J. Vet. Med. Sci.*, 64(2): 95-100 (2002).
27. S.L. LAUREN, T. ARAKAWA, K. STONEY, M.F. ROHDE, Covalent dimerization of recombinant human interferon-gamma. *Arch. Biochem. Biophys.*, 306(2): 350-353 (1993).
28. R. MIRONOVA, T. NIWA, R. DIMITROVA, M. BOYANOVA, I. IVANOV, Glycation and post-translational processing of human interferon-gamma expressed in Escherichia coli. *J. Biol. Chem.*, 278(51): 51068-51074 (2003).
29. T. SARENEVA, J. PIRHONEN, K. CANTELL, N. KALKKINEN, I. JULKUNEN, Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma. Biochem. J., 303(3): 831-840 (1994).
30. S. RUF, M. HERMANN, I.J. BERGER, H. CARRER, R. BOCK, Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotech.*, 19(9): 870-875 (2001).
31. E. RINDERKNECHT, B. O'CONNOR, H. RODRIGUEZ, Natural human interferon-gamma. Complete amino acid sequence and determination of sites of glycosylation. *J. Biol. Chem.*, 259(11): 6790-6797 (1984).
32. D. BOYHAN, H. DANIELL, Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant. Biotecnol. J.*, 9(5): 585-598 (2011).
33. H. DANIELL, A. DHINGRA, Multigene engineering: dawn of an exciting new era in biotechnology. *Curr. Opin. Biotech.*, 13(2): 136-141 (2002).
34. Y. ARAI, T. SHIKANAI, Y. DOI, S. YOSHIDA, I. YAMAGUCHI, H. NAKASHITA, Production of poly-hydroxybutyrate by polycistronic expression of bacterial genes in tobacco plastid. *Plant. Cell. Physiol.*, 45(9): 1176-1184 (2004).
35. O. DRECHSEL, R. BOCK, Selection of Shine-Dalgarno sequences in plastids. *Nucl. Acids. Res.*, 39(4): 1427-1438 (2011).