



Overexpression of the recombinant human interferon-beta (*rhIFN-β*) gene in tobacco chloroplasts

ASGHAR FEIZI, BAHRAM BAGHBANKOHNHROUZ*

Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Iran

Abstract

Chloroplast genetic engineering is a convenient method for the production of recombinant proteins by increasing the expression level of transgenes. Interferon-beta (IFN- β) is a member of type I interferons that possess some pharmaceutical properties. The present study aimed to investigate the overexpression and production of the recombinant human IFN- β gene (*rhIFN-β*) in the tobacco chloroplast genome. For this purpose, a codon-optimized *rhIFN-β* was transferred to the pVSR326 plastid vector containing the *aadA* gene as a selectable marker. The *rhIFN-β* gene was then successfully introduced into the tobacco chloroplast genome by using a gene gun. The integration of the *rhIFN-β* gene into the chloroplast genome and the homoplasmy of the T1 progeny were confirmed by PCR and Southern blot analysis, respectively. RT-PCR and western blot analyses confirmed the transcription and translation of the *rhIFN-β* gene, respectively. An enzyme-linked immunosorbent assay (ELISA) showed that the rhIFN- β protein in transplastomic plants comprised approximately 2.4% of total soluble protein (TSPs). The bioassay confirmed that the rhIFN- β protein expressed in the tobacco chloroplast had a relatively high biological activity (2.9×10^4 IU/ml) and protected human amniotic cells against the vesicular stomatitis virus (VSV). On the basis of these findings, it can be concluded that plastid transformation can serve as an operative method for the production of pharmaceutical recombinant proteins.

Key words: plastid expression, chloroplast engineering, homoplasmy, pharmaceutical protein, molecular farming

Introduction

To produce recombinant proteins in large amounts, suitable plants can be used as a convenient alternative to mammalian species, insects, yeast cell cultures, and microbial industrial bioreactors. Tobacco is characterized by a high production of biomass (almost 170 tons per hectare), high seed yield, self-pollination, maternal chloroplast inheritance, and relatively easy genetic engineering as compared to other plants; thus, it constitutes a good option for molecular farming and production of pharmaceutical recombinant proteins (Ivanov et al., 2020). Compared to other systems, the production of pharmaceutical proteins through plant chloroplast genetic engineering has many advantages such as a high level of recombinant protein accumulation in chloroplasts due to their polyploidy nature, limited number of protein degradation pathways, lack of side effects, and

silencing because of homologous directional integration of transgene in specific regions in the chloroplast genome and maternal heritage of chloroplast genes (Abd-Aziz et al., 2020; Kopertekh and Schiemann, 2019). Furthermore, the prokaryotic expression system of the chloroplasts enables researchers to introduce multiple genes within an operon to express an entire metabolic pathway (Schindel et al., 2018).

Interferon-beta (IFN- β) is a member of type I interferons (IFNs). It is known for its antiviral and immunomodulatory characteristics (Morowvat et al., 2014). IFN- β is one of the main therapies for treating multiple sclerosis (MS), although many patients cannot afford this treatment because of its high cost (Thompson et al., 2013). Because of the clinical success of IFN- β , there is a growing global demand for this important product (McCrae, 2021; Palesh et al., 2008; Wood, 2020). To res-

* Corresponding author: Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Iran; e-mail: bbaghbankohnehouz@gmail.com

pond to this increasing demand, a cheaper production system is required to be developed. The production of pharmaceutical recombinant proteins using transgenic plants is an economical and effective method. Compared to the expression systems for recombinant proteins using nuclear transformation systems, the chloroplast expression system offers several advantages, including high expression level, occurrence of the position effect, maternal inheritance that lessens the risk of dispersal of the transgene into the environment, ability to express polycistronic genes, and no obvious gene silencing (Karimi et al., 2013; Sheikh-Mohamadi et al., 2017; Sheikh Mohamadi et al., 2018; Sheikh Beig Goharrizi et al., 2016). Svab et al. (1990) developed a stable transformation protocol for tobacco chloroplasts by using the gene gun method. In the past two decades, remarkable progression has been made in producing valuable recombinant proteins using plant chloroplast expression systems (Adem et al., 2017), for example, the production of monoclonal antibody (Fischer et al., 2004), stable antibiotic protein (Oey et al., 2009), interferon-alpha (Arlen et al., 2007), and recombinant single-domain monoclonal antibody (Rajabi-Memari et al., 2006).

Model plants exhibit several properties such as easy transformability, short life cycle, high seed production, and simple propagation by seeds, which make them appropriate for use in functional genomics (Dejahang et al., 2018; Farajpour et al., 2011). Several methods are available to transform chloroplasts, such as transformation by polyethylene glycol and gene gun methods. Polyethylene glycol is used for plant species with chloroplasts that can be extracted and regenerated (Bock, 2014). Simplicity and the ability to transform different plant species make gene gun transformation a convenient and popular method (Adem et al., 2017). It has been assumed that the engineering of tobacco chloroplasts is an efficient and cost-effective method to produce IFN- β . Therefore, the present study aimed to produce recombinant human interferon-beta (rhIFN- β) by expression in tobacco chloroplasts.

Materials and methods

Plant materials and growth conditions

Green leaves of *Nicotiana tabacum* L. cv. "Samsun" were used for chloroplast transformation. The tobacco seeds were obtained from the National Plant Gene-bank,

Karaj, Iran. They were sterilized by 70% ethanol for 1 min, incubated in 2% sodium hypochlorite for 15 min, and cultured on MS medium containing 30 g and 8 g/l of sucrose and agar, respectively. The pH of the medium was adjusted to 5.6. The cultures were kept under 16 h photoperiod ($40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) provided by 40-W white fluorescent lamps at $25 \pm 2^\circ\text{C}$ for 3–4 weeks. The green leaves were then transferred to a Revised Medium for Organogenesis of *Nicotiana plumbaginifolia* (RMOP) medium one day before gene bombardment (Scotti et al., 2009).

Plasmid preparation

The tobacco chloroplast codon-optimized *ifn- β* gene was developed by ShineGene Molecular Biotech Inc. (Shanghai, China). The *ifn- β* codon sequence was optimized by GenScript's patented OptimumGene™ algorithm. The pVSR326 chloroplast vector, which was kindly provided by Dr. Vanga Siva Reddy (International Centre for Genetic Engineering and Biotechnology, New Delhi, India), was used for plastid transformation. The vector is capable of integrating the desired foreign genes into the tobacco chloroplast genome by a site-specific integration between the *rbcL* and *ccD* genes (nucleotides 58 056–60 627). The *NcoI* and *SacI* restriction enzymes were used for inserting the subclone *ifn- β* coding sequence into the pVSR326 vector according to a method described by Reddy et al. (2002). The integration of the *ifn- β* gene was verified by excising it using the above-mentioned restriction enzymes and sequencing the obtained sequence. The recombinant plasmid was termed pVSR326-IFN β . One positive clone was selected for further experiments (Karimi et al., 2013). The *aadA* gene was used as a selectable marker that provided spectinomycin resistance. The rice *psbA* promoter and terminator were used in the recombinant pVSR326-IFN- β plasmid to control the expression of the *ifn- β* gene (Reddy et al., 2002). The GGAGTC ribosome binding site was used for enhancing the translation upstream of the *ifn- β* coding sequence. The QIAGEN Plasmid Midi Kit (QIAGEN, Germany) was used for the isolation of plasmid DNA according to the manufacturer's protocol.

Plant transformation and regeneration

The PDS-1000He (Bio-Rad, Hercules, CA, USA) gene gun with 900 psi was used for the transformation of tobacco leaves through the gene bombardment method. It involved using the pVSR326-IFN- β plasmid DNA-co-

ated gold (0.6 microns) micro-projectile (Daniell, 1997). Nearly 100 tobacco leaves were bombarded with the prepared plasmid. Another 100 tobacco leaves were transformed with the native *Agrobacterium tumefaciens* without any constructs as control for bioactivity assay.

After bombardment, the leaves were placed on the RMOP medium and incubated in dark at 25°C for 48 h under a 16:8-h photoperiod. The bombarded leaves were cut into 5×5 mm pieces and cultured in RMOP medium containing NAA (0.1 mg/l), BA (1 mg/l), and spectinomycin dihydrochloride (500 mg/l). They were left until a suitable time for the selection and regeneration of transplastomic tobacco cells. Six to eight weeks later, putative transplastomic shoots were identified as resistant to spectinomycin. Three rounds of regeneration involved using small sections of the shoots as explants on the RMOP medium containing spectinomycin. The antibiotic-resistant shoots were further cultured on the basic MS medium in the growth chamber with the 16:8-h light/dark photoperiod at 25°C. The rooted transplastomic plants were potted and continued to grow in a growth room at 26°C and a 16:8-h photoperiod.

PCR analysis of transplastomic plants

DNA was extracted from the leaves of putative transplastomic plants and their wild counterparts by an i-genomic DNA extraction mini kit (iNtRON Biotechnology, Korea) according to the manufacturer's protocol. A PCR analysis was performed to confirm the integration of the *ifn- β* and *aadA* genes into the chloroplast genome. The *ifn- β* forward (5'-CGACAAGATTCTTCTTCTACTGGATGG-3') and reverse primers (5'-GTCGACATTACGAGATAACCTGTCA-3') were used for PCR analysis. The following forward (5'-GGATCCATGGCTCGTGAA GCGGT-3') and reverse (5'-CCATGGATCCCTCCCTT TATTTGCCAACTACCT-3') primers were used for ensuring the integration of *aadA* in transplastomic plants.

The PCR reaction mixture contained 1× PCR buffer, 0.2 mM dNTP mixture, 1 μ M of each primer, one unit of Taq DNA polymerase, and 1 μ l digested DNA in a total volume of 20 μ l. The following PCR conditions were used: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 58°C, and 1 min 72°C, and a final extension of 5 min at 72°C. The PCR products were then separated by electrophoresis on a 1% agarose gel. The gels were stained with ethidium bromide and visualized by ultraviolet illumination.

Southern blot analysis

Southern blot analysis was performed to analyze the integration of the *ifn- β* gene into the tobacco chloroplast genome. Twenty micrograms of genomic DNA from the transplastomic and non-transplastomic plants were digested by 10 units of *Cla*I in CutSmart buffer (New England BioLabs, USA) at 37°C overnight. The products were separated by electrophoresis on a 1% agarose gel and were blotted on the Hybond N membrane (Amersham, Buckinghamshire, UK) according to the standard procedure described by Sambrook Russel (2001). The detection of the *ifn- β* cassette was carried out by a 453-bp digoxigenin (DIG)-labeled probe (P1) that was produced by the PCR DIG Labeling MIX (Roche, Germany) using *ifn- β* as a template and specific forward (5'-CGACAAGATTCTTCTTCTACTGGATGG-3') and reverse (5'-GTCGACATTACGAGATAACCTGTCA-3') primers following the manufacturer's instructions.

The homoplasty of transplastomic plants was confirmed by an 820-bp DIG-labeled *rbcl* probe (P2) using the following specific forward (5'-CTCACAACCATTTATGCG-3') and reverse (5'-GTGGACGTTTTGGATAG-3') primers and tobacco DNA as a template according to the procedure described by Karimi et al. (2013). Hybridization and detection were performed according to the manufacturer's instructions (Roche).

Transcriptional and translational analyses

RT-PCR was performed for analyzing the transcription of the *ifn- β* gene. In the first step, total RNA was isolated from the transplastomic and wild-type plant leaves using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The RT-PCR reaction was performed in a final volume of 20 μ l containing the gDNA Wipeout Buffer, RNase-free water, Quantiscript Reverse Transcriptase, RT primer mix, Quantiscript RT Buffer, and 1 μ g of total RNA. The reaction was performed in an MJ Research Thermal Cycler under the following conditions: 2 min at 42°C for gDNA elimination, immediately followed by placing the reaction tube on ice for adding the reaction mixture, 30 min at 42°C for reverse transcription, and 3 min at 95°C for enzyme inactivation. The presence and expression level of *ifn- β* were determined by the RT-PCR analysis. The RT-PCR conditions and primers were the same as mentioned above, except that cDNA was used instead of DNA template. The RT-PCR product was separated by

electrophoresis on a 1% agarose gel and then stained with 0.5 mg/l ethidium bromide. The PCR products were visualized under UV illumination. A 1-kb DNA ladder (Fermentas) was used as the size marker.

SDS-PAGE and western blot analyses were used to confirm translation of the *ifn-β* gene. The isolation of total proteins from the leaves, together with SDS-PAGE and immunoblotting procedures, were performed as described previously by Karimi et al. (2013). Fifty micrograms of TSP was separated on a 10% SDS-PAGE. The bands were transferred to a nitrocellulose membrane (Roche) by using a Mini Trans-Blot electrophoretic transfer cell (BioRad, USA) in transfer buffer (50 mM Tris base, 40 mM glycine, 0.04% SDS, and 20% methanol, pH 8.3). The membrane was washed three times in PBST buffer, and the color was developed using a horseradish peroxidase (HRP) staining solution (DAB; Sigma). The chromogenic reaction was stopped by rinsing the membrane twice with distilled water.

The expression of the *ifn-β* recombinant gene was quantified in transplastomic plants by ELISA. Fully grown leaves from transplastomic and wild-type plants were ground with liquid nitrogen and homogenized in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄). An ELISA test was performed using commercial anti-IFN-β antibodies (Sigma), where the recombinant IFN-β (Sigma, St. Louis, MO, USA) served as the standard as described previously (Wirth et al. 2004).

Leaf extract preparation

For the leaf extract preparation, fresh leaves were air-dried for 5 days at 30 °C, and 100 g of dried material was macerated in 2 l of distilled water. The extract was decanted after 24 h. The filtrate was dried in an oven at 40 °C for 4 days. The dried extract was weighed and dissolved in distilled water to obtain a 200 mg/ml stock concentration.

Bioactivity assay of rhIFN-β

The bioactivity assay for rhIFN-β was performed based on cytoplasmic inhibition of vesicular stomatitis virus (VSV) in human amnionic cells (WISH), as described by Langford et al. (1981). Human amnionic cells were seeded onto a 96-well tissue culture plate and incubated at 37 °C overnight. After a confluent monolayer of cells was formed, approximately 100 μl of the hIFN-β standard (Sigma-Aldrich, Germany) was added to the wells

along with the addition of leaf extracts of the rhIFN-β that had been purified. The extract of leaves infiltrated with native *A. tumefaciens* without any constructs was considered as the negative control, whereas the standard hIFN-β was used as the positive control. Serial half-log₁₀ dilutions (10^{-0.5}) of protein samples were used. Each sample was assayed in three replicates. The plate was incubated at 37 °C for 16 h. The medium was then disposed of, and the cells were washed twice with sterile phosphate-buffered saline (PBS). Next, 100 μl (100 TCID₅₀) VSV was inoculated into each well, and the plate was incubated at 37 °C. Virus infection was visually checked, and the plate was incubated for 30 h until all cells that had not received the IFN-β treatment were infected by VSV. The IFN-β activity was measured in international units (IU) per mg of TSP of tobacco leaf according to the method of Langford et al. (1981). One unit of IF is present in the dilution of a sample that inhibits the formation of plaques equal to 50% of that in the virus control.

Results

Plant transformation and regeneration

The tobacco chloroplast codon-optimized *ifn-β* gene sequence (509 bp) was deposited in the GeneBank (*MH285815.1*). The recombinant gene contained an *ifn-β1a* coding sequence, lacking 21 amino acids from the protein N-terminus. The rice *psbA* promoter and terminator were used as transcriptional elements in pVSR326-IFN-β plasmids, while the *aadA* gene was used as a selectable marker that provided spectinomycin resistance. The ribosome binding site 5'–GGAGTC–3' was used for enhancing the translation upstream of the *ifn-β* coding sequence. The *rbcl-accD* flanking sequences integrated the transgene cassette (6.4 kb) into the chloroplast genome between the *rbcl-accD* region through two homologous recombination (Fig. 1A) (Reddy et al., 2002). The 25 antibiotic-resistant green calli were obtained from approximately 100 tobacco leaves that were bombarded by the DNA of pVSR326-IFN-β. The antibiotic-resistant calli were regenerated on an RMOP medium (Fig. 1B and 1C). The transplastomic lines were regenerated four times on the selection medium, and after 8 months, they were transferred to MS medium containing 500 mg/l of spectinomycin, so that rooting occurred before the transfer to a growth chamber. All 25 antibiotic-resistant green calli were successfully regene-

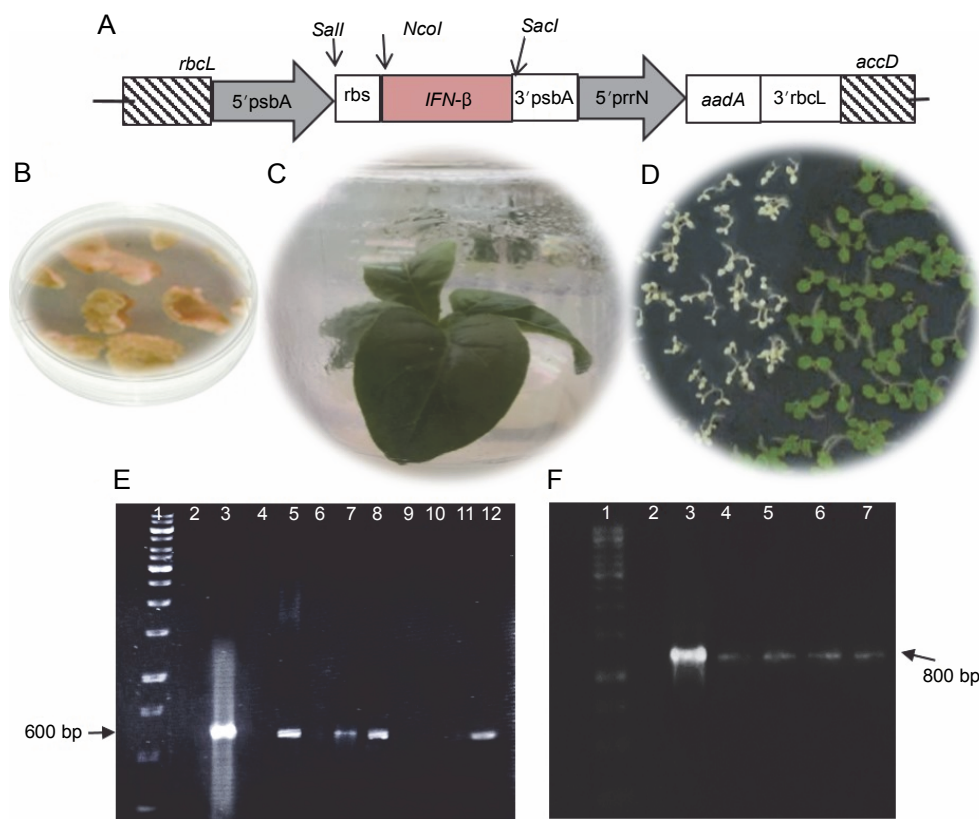


Fig. 1. A) Schematic structure of the recombinant *ifn-β* gene in the pVSR326 chloroplast transformation vector: the *aadA* spectinomycin resistance gene was used as a selectable marker; 5'prn rice 16SrRNA operon promoter; Rbs ribosome binding site; B) spectinomycin-resistant green calli; C) transplastomic regenerated plant; D) germination of transplastomic T1 (green) and wild-type (white) seeds; E) PCR results of the putative transplastomic plants with *ifn-β* primers (arrows); F) detection of putative transplastomic plants using *aadA* primers

rated and rooted on MS medium containing 500 mg/l spectinomycin. The seeds of the regenerated plants were cultured on a medium containing spectinomycin and were assessed for stability regarding the integration of *ifn-β* in transplastomic plants (Fig. 1D).

PCR and Southern blot analyses of transplastomic plants

PCR using the *ifn-β* and *aadA* specific primers was performed for 25 spectinomycin-resistant shoots, among which 4 showed both the 509 and 800 bp bands, representing the integration of the *ifn-β* and *aadA* genes, respectively (Fig. 1E and 1F).

To verify the plant homoplasmy and the correct integration of the cassette between the *rbcl* and *accD* genes, DNA blot analysis was performed on PCR-positive transplastomic lines. In the chloroplast genome, the *Clal* enzyme was used to cut the 5' and 3' borders of *rbcl* and *accD* sequences, respectively (Fig. 2A and 2B). The digestion of chloroplast DNA with the *Clal* enzyme formed

a 6.4-kb hybridizing band by a DIG-labeled *ifn-β* probe, which confirmed the insertion of *ifn-β* into the transplastomic chloroplast genome. The wild-type plants showed no detectable band (Fig. 2C). Homoplasmy was confirmed using the *rbcl* probe, which identified the 6.4 and 3.4 kb bands in transplastomic and wild-type plants, respectively (Fig. 2D).

Transcription and translation analyses of transplastomic plants

RT-PCR and western blot assays were performed to identify the transcription and translation products of *ifn-β* in transplastomic plants. RT-PCR results were obtained using *ifn-β*-specific primers and showed an approximately 550 bp *ifn-β* mRNA, thereby confirming the transcription of the *ifn-β* gene in the chloroplasts of transplastomic lines, whereas no band was detected in wild-type plants (Fig. 3A). Western blotting assay with the IFN-β-specific antibody detected the IFN-β protein.

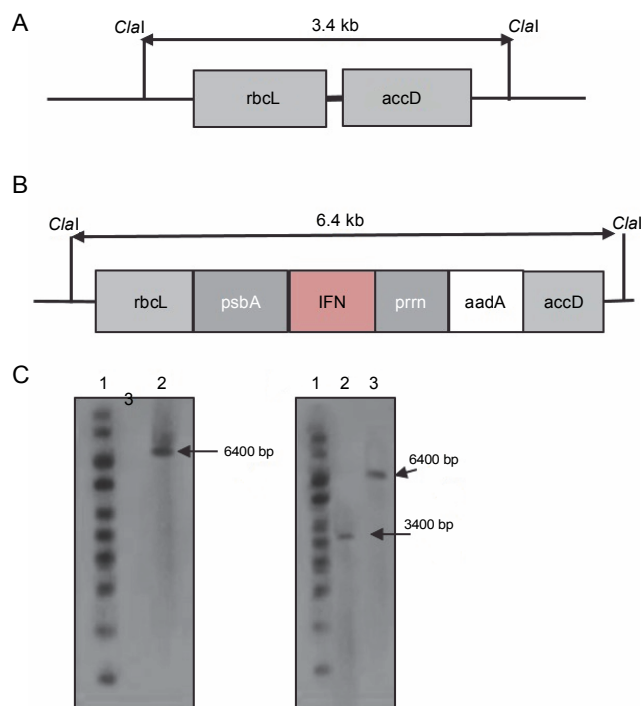


Fig. 2. Southern blot analysis of the tobacco transplastomic line. A) graphic presentation of the *Cla*I restriction scheme in the wild-type chloroplast genome; B) graphic presentation of the *Cla*I restriction scheme in the transplastomic tobacco chloroplast genome; C) lane 1 – 1-kb DNA ladder, lane 2 – non-transplastomic control plant DNA, lane 3 – transplastomic line; the *ifn- β* probe was hybridized to a 6.4-kb fragment in the transplastomic line; D) lane 1 – 1-kb DNA ladder, lane 2 – wild-type plant DNA sample, lane 3 – plant DNA sample of the transplastomic line; the *rbcL* probe hybridized to a 7.4-kb fragment in the transplastomic line and to a 3.4-kb fragment in the wild-type plant

The blotting assay showed an approximately 25 kDa band in the transplastomic plants, indicating the production of the IFN- β protein in the chloroplast of these plants, while no bands were observed in the wild-type plants (Fig. 3B).

The ELISA assay was used to quantify the IFN- β protein in transplastomic plants. The absorbance readings of the samples were compared using the diluted grades of the IFN- β standard antigen. The IFN- β TSP percentage was calculated using the Bradford method (Sambrook and Russel, 2001). The percentage of expressed IFN- β was calculated as the percentage of TSP by using the Bradford assay. The amount of the IFN- β protein was approximately 2.4–2.5% of TSP (Fig. 4).

Bioactivity assay of rhIFN- β

The wells with sample cells and the standard hIFN- β showed normal growth after 30 h of infection with the

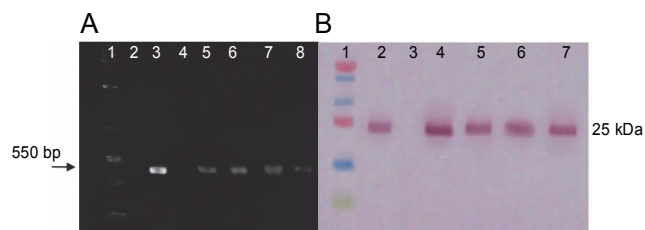


Fig. 3. RT-PCR and western blotting assay results. A) RT-PCR analysis performed on leaves of transplastomic lines and wild-type plants by using *ifn- β* -specific primers; lane 1 – PCR product without cDNA template (negative control), lane 2 – 1-kb DNA ladder (Fermentas), lane 3 – total RNA as the template, lane 4 – PCR product from pVSR326-IFN β DNA as the template (positive control), lane 5 – DNA of the wild-type plant, lanes 6–9 – transplastomic lines; B) the IFN- β protein blotting assay in transplastomic lines by using anti-IFN- β antibody: lane 1 – protein molecular weight marker (Fermentas), lane 2 – positive control, lane 3 – 20 μ g of TSP from wild-type plants, lane 4–7 – 20 μ g TSP from transplastomic lines

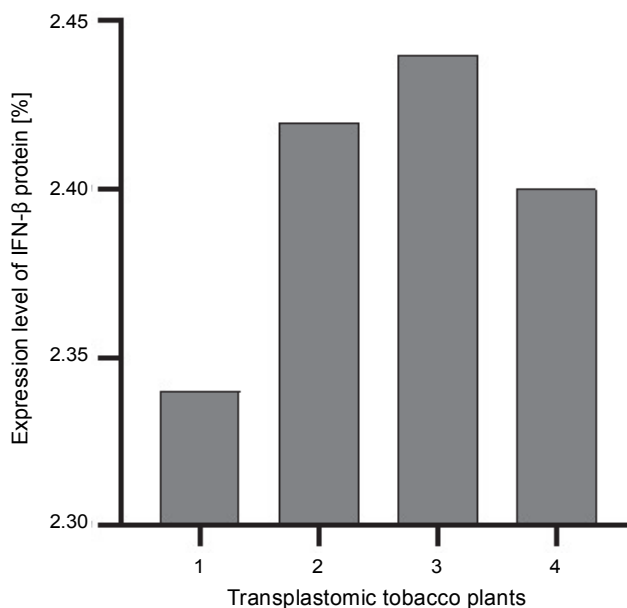


Fig. 4. IFN- β quantification in transplastomic tobacco plants; the amount of IFN- β in four independent transgenic plants as compared to the total soluble protein is shown; vertical bars show standard deviation (SD) of mean values ($n = 3$)

VSV, while the control wells containing the extract of leaves with native *A. tumefaciens* without any constructs exhibited severe pathological changes as they became necrotic and did not show normal growth. By using hIFN- β as the standard, the bioactivity of the recombinant rhIFN- β was measured to be 2.9×10^4 IU/mg of TSP. The bioactivity assay showed that the rhIFN- β could protect human amnionic cells against VSV.

Discussion

Plastid genetic engineering in plants has significantly progressed during the past decades and has become a promising method for the production of high-value metabolites and proteins, e.g., industrial enzymes, biopharmaceuticals, and diagnostic reagents (Clarke et al., 2013; De Marchis et al., 2012; van Eerde et al., 2019; Younessi-Hamzekhanlu et al., 2021). The diverse features of chloroplast transformation include the dynamic concept of site-specific integration of the transgene into the plastid genome (Limaye et al., 2006), lack of position effects and epigenetic gene silencing (Daniell et al., 2001; Rigano et al., 2013; Ruhlman et al., 2010), insertion of transgenes in operons (Lu et al., 2013), and the biosafety profile of transplastomic plants. These qualities make chloroplast transformation an appropriate method for use in plant biotechnology. Currently, many recombinant proteins are expressed in chloroplasts, and efforts have been made to produce different pharmaceutical proteins in plant chloroplast (Hoelscher et al., 2018; Razmi et al., 2019; van Eerde et al., 2019). In the present study, the codons of the *ifn β* coding sequence were optimized for expression in tobacco chloroplasts (Krasniqi et al., 2018). The production of the *rhIFN β* protein in tobacco chloroplasts was confirmed by protein blot analysis using *IFN β* -specific antibodies, where, as expected, a protein of approximately 25 kDa was detected.

A high level of expression of foreign proteins in plastids is a major development in plant biotechnology. It makes plastid transformation as an ideal approach for large-scale production of recombinant proteins (Gao et al., 2012). It has been reported that the expression of the HIV entry inhibitor griffithsin in the tobacco protoplast exceeded 5% of the plant's TSP (Hoelscher et al., 2018). A simple purification process of plastidic griffithsin from the leaves proved this to be a feasible method. Moreover, the protein was characterized by a high level of virus neutralization activity as compared to the activity of the protein expressed in bacteria (Hoelscher et al., 2018). The dried tobacco leaves also serve as a storable source material for large-scale production of the recombinant griffithsin protein (Hoelscher et al., 2018). Arlen et al. (2007) documented a high level (up to 20% of TSP) of *IFN α 2b* production in tobacco chloroplasts. The transgenic *IFN α 2b* showed similar *in vitro*

and *in vivo* biological activities as compared to commercial products. Previous studies have reported different expression levels of recombinant proteins in the tobacco chloroplast, such as interferon alpha-5 at the level of 4,372 pg/g fresh weight (Khan and Nurjis, 2012) and *Bacillus anthracis* 83-kDa protective antigen (PA) at 18% of TSP (Watson et al., 2004). Our results showed that the expression level of the recombinant protein was relatively low, which was in agreement with earlier studies (Razmi et al., 2019; Schindel et al. 2018; Shen et al., 2010). Many studies have indicated that the low expression level of recombinant proteins in chloroplasts could be attributed to codon usage, translation efficiency, promoter, and protein stability in chloroplasts (Ahmad et al., 2020; Apel et al., 2010; De Marchis et al., 2012; Rowland et al., 2015; Soria-Guerra et al., 2009; Younessi-Hamzekhanlu et al., 2020). Huy Kim (2019) reported a low expression level (0.07% of TSP) of the porcine epidemic diarrhea antigen in tobacco, whereas fusion with a stable B subunit of the cholera toxin protein increased the expression level by up to 1.4% TSP. However, several researchers have claimed that the mechanism of protein instability is still unclear and the prediction of foreign protein stability in plastids was not possible (Hoelscher et al., 2018). Razmi et al. (2019) expressed *ifn γ* in the tobacco chloroplast at 0.42% of the TSP, which was much higher than its expression in nuclear-transformed plants (0.03%). These results could be ascribed to the lack of post-translational modifications in chloroplasts. Although the human *IFN β* protein has an N-glycosylation site, the *IFN β* protein expressed in transplastomic plants had almost the same size as that expressed in *Escherichia coli* (Fig. 3); this is because N-glycosylation is not known to occur within chloroplasts (Daniell et al., 2009). Chloroplasts are capable of performing some post-translational modifications such as folding, formation of disulfide bonds, and lipid modification, but they do not support glycosylation (Razmi et al., 2019).

The bioassay analysis confirmed the high bioactivity (2.9×10^4 IU/ml) of *rhIFN β* expressed in tobacco chloroplasts. The protein could protect human amnionic cells against VSV infection. Previous studies have shown the bioactivity of *rhIFN γ* in protecting monkey Vero cells against VSV (Heidari-Japelaghi et al., 2020), which is in agreement with our results. Even though previous stu-

dies have confirmed the *in vitro* and *in vivo* biological activities of recombinant pharmaceutical proteins in chloroplasts, we propose future studies to examine the biological activity of IFN- β following its expression in tobacco chloroplasts. In the present study, the recombinant IFN- β protein was successfully expressed at a reasonable level in the tobacco chloroplast, which may lead to the cost-effective production of IFN- β .

Conclusion

In the present study, the tobacco chloroplast transformation method was used to produce human IFN- β , which is known for its antiviral and immunomodulatory activities. It can be successfully used in treating MS. The integration of the *ifn*- β gene with the chloroplast genome was confirmed by molecular techniques at the DNA, RNA, and protein levels. The bioassay confirmed that the recombinant IFN- β had relatively high bioactivity in tobacco chloroplasts, as revealed by its capability to protect the human amnionic cells against VSV infection. Future experiments will determine the effect of *ifn* gene expression.

References

- Abd-Aziz N., Tan B.C., Rejab N.A., Othman R.Y., Khalid N. (2020) *A new plant expression system for producing pharmaceutical proteins*. Mol. Biotechnol, 62: 240–251.
- Adem M., Beyene D., Feyissa T. (2017) *Recent achievements obtained by chloroplast transformation*. Plant Meth. 13: 1–11.
- Ahmad N., Mehmood M.A., Malik S. (2020) *Recombinant protein production in microalgae: emerging trends*. Protein Peptide Lett. 27: 105–110.
- Apel W., Schulze W.X., Bock R. (2010) *Identification of protein stability determinants in chloroplasts*. Plant J. 63: 636–650.
- Arlen P.A., Falconer R., Cherukumilli S., Cole A., Cole A.M., Oishi K.K., Daniell H. (2007) *Field production and functional evaluation of chloroplast derived interferon α 2b*. Plant Biotechnol. J. 5: 511–525.
- Bock R. (2014) *Genetic engineering of the chloroplast: novel tools and new applications*. Curr. Opin. Biotechnol. 26: 7–13.
- Clarke J.L., Waheed M.T., Lössl A.G., Martinussen I., Daniell H. (2013) *How can plant genetic engineering contribute to cost-effective fish vaccine development for promoting sustainable aquaculture?* Plant Mol. Biol. 83: 33–40.
- Daniell H. (1997) *Transformation and foreign gene expression in plants mediated by microprojectile bombardment*. [in:] *Recombinant gene expression protocols*. Springer: 463–489.
- Daniell H., Lee S.-B., Panchal T., Wiebe P.O. (2001) *Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts*. J. Mol. Biol. 311: 1001–1009.
- Daniell H., Singh N.D., Mason H., Streatfield S.J. (2009) *Plant-made vaccine antigens and biopharmaceuticals*. Trends Plant Sci. 14: 669–679.
- De Marchis F., Pompa A., Bellucci M. (2012) *Plastid proteostasis and heterologous protein accumulation in transplastomic plants*. Plant Physiol. 160: 571–581.
- Dejahang A., Mahna N., Farsad-Akhtar N., Mousavi A. (2018) *Identification, isolation and in silico characterization of Fragaria vesca homologue of tempranillo gene*. Bionature 38(6): 337–346.
- Farajpour M., Ebrahimi M., Amiri R., Noori S., Sanjari S., Golzari R. (2011) *Study of genetic variation in yarrow using inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers*. Afr. J. Biotechnol. 10: 11137–11141.
- Fischer R., Stoger E., Schillberg S., Christou P., Twyman R.M. (2004) *Plant-based production of biopharmaceuticals*. Curr. Opin. Biotechnol. 7: 152–158.
- Gao M., Li Y., Xue X., Wang X., Long J. (2012) *Stable plastid transformation for high-level recombinant protein expression: promises and challenges*. J. Biomed. Biotechnol. 2012: 158232.
- Heidari-Japelaghi R., Valizadeh M., Haddad R., Dorani-Uliaie E., Jalali-Javaran M. (2020) *Production of bioactive human IFN- γ protein by agroinfiltration in tobacco*. Protein Expr. Purif. 173: 105616.
- Hoelscher M., Tiller N., Teh A.Y.-H., Wu G.-Z., Ma J.K., Bock R. (2018) *High-level expression of the HIV entry inhibitor griffithsin from the plastid genome and retention of biological activity in dried tobacco leaves*. Plant Mol. Biol. 97: 357–370.
- Huy N.-X., Kim M.-Y. (2019) *Improved expression of porcine epidemic diarrhea antigen by fusion with cholera toxin B subunit and chloroplast transformation in Nicotiana tabacum*. Plant Cell Tissue Organ. Cult. 137: 213–223.
- Ivanov N.V., Sierro N., Peitsch M.C. (2020) *The tobacco plant genome*. 1st ed. Springer, Cham, Switzerland. 1: 263.
- Karimi F., Mousavi A., Salmanian A.H., Alizadeh H., Rafati S. (2013) *Immunogenicity of EIT chimeric protein expressed in transplastomic tobacco plants towards development of an oral vaccine against Escherichia coli O157: H7*. Plant Biotechnol. Rep. 7: 535–546.
- Khan M.S., Nurjis F. (2012) *Synthesis and expression of recombinant interferon alpha-5 gene in tobacco chloroplasts, a non-edible plant*. Mol. Biol. Rep. 39: 4391–4400.
- Kopertekh L., Schiemann J. (2019) *Transient production of recombinant pharmaceutical proteins in plants: evolution and perspectives*. Curr. Med. Chem. 26: 365–380.
- Krasniqi A., D’Huyvetter M., Devoogdt N., Frejd F.Y., Sørensen J., Orlova A., Keyaerts M., Tolmachev V. (2018) *Same-day imaging using small proteins: clinical experience and translational prospects in oncology*. J. Nucl. Med. 59: 885–891.

- Langford M.P., Weigent D., Stanton G.J., Baron S. (1981) *Virus plaque-reduction assay for interferon: microplaque and regular macroplaque reduction assays*. [in:] *Methods in enzymology*. Elsevier: 339–346.
- Limaye A., Koya V., Samsam M., Daniell H., Limaye A., Koya V., Samsam M., Daniell H. (2006) *Receptor-mediated oral delivery of a bioencapsulated green fluorescent protein expressed in transgenic chloroplasts into the mouse circulatory system*. *FASEB J.* 20: 959–961.
- Lu Y., Rijzaani H., Karcher D., Ruf S., Bock R. (2013) *Efficient metabolic pathway engineering in transgenic tobacco and tomato plastids with synthetic multigene operons*. *Proc. Natl Acad. Sci. USA* 110: E623–E632.
- McCrae C. (2021) *INEXAS: a phase 2 randomised trial of on-demand inhaled interferon beta-1a in severe asthmatics*. *Clin. Exp. Allergy* 51(2): 273–283.
- Morowvat M.H., Babaeipour V., Rajabi-Memari H., Vahidi H., Maghsoudi N. (2014) *Overexpression of recombinant human beta interferon (*rhINF- β*) in periplasmic space of *Escherichia coli**. *Iran. J. Pharm. Res.* 13: 151.
- Oey M., Lohse M., Kreikemeyer B., Bock R. (2009) *Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic*. *Plant J.* 57: 436–445.
- Palesh M., Jonsson P.M., Jamshidi H., Wettermark B., Tomson G., Fredrikson S. (2008) *Diffusion of interferon beta in Iran and its utilization in Tehran*. *Pharmacoepidemiol. Drug Saf.* 17: 934–941.
- Rajabi-Memari H., Jalali-Javaran M., Rasaei M., Rahbarizadeh F., Forouzandeh-Moghadam M., Esmaili A. (2006) *Expression and characterization of a recombinant single-domain monoclonal antibody against MUC1 mucin in tobacco plants*. *Hybridoma* 25: 209–215.
- Razmi S., Javaran M.J., Bagheri A., Honari H., Zadeh M.S. (2019) *Expression of human interferon gamma in tobacco chloroplasts*. *Rom. Biotechnol. Lett.* 24: 208–215.
- Reddy V.S., Leelavathi S., Selvapandiyani A., Raman R., Giovanni F., Shukla V., Bhatnagar R.K. (2002) *Analysis of chloroplast transformed tobacco plants with *cryIIa5* under rice *psbA* transcriptional elements reveal high level expression of Bt toxin without imposing yield penalty and stable inheritance of transplastome*. *Mol. Breed.* 9: 259–269.
- Rigano M.M., De Guzman G., Walmsley A.M., Frusciante L., Barone A. (2013) *Production of pharmaceutical proteins in solanaceae food crops*. *Int. J. Mol. Sci.* 14: 2753–2773.
- Rowland E., Kim J., Bhuiyan N.H., van Wijk K.J. (2015) *The Arabidopsis chloroplast stromal N-terminome: complexities of amino-terminal protein maturation and stability*. *Plant Physiol.* 169: 1881–1896.
- Ruhlman T., Verma D., Samson N., Daniell H. (2010) *The role of heterologous chloroplast sequence elements in transgene integration and expression*. *Plant Physiol.* 152: 2088–2104.
- Sambrook J., Russel D.W. (2001) *Molecular cloning: a laboratory manual*. Ed. J. Sambrook, D.W. Russell. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory.
- Schindel H.S., Piatek A.A., Stewart C.N., Lenaghan S.C. (2018) *The plastid genome as a chassis for synthetic biology-enabled metabolic engineering: players in gene expression*. *Plant Cell Rep.* 37: 1419–1429.
- Scotti N., Alagna F., Ferraiolo E., Formisano G., Sannino L., Buonaguro L., De Stradis A., Vitale A. et al. (2009) *High-level expression of the HIV-1 Pr55 gag polyprotein in transgenic tobacco chloroplasts*. *Planta* 229: 1109–1122.
- Sheikh-Mohamadi M.-H., Etemadi N., Nikbakht A., Farajpour M., Arab M., Majidi M.M. (2017) *Screening and selection of twenty Iranian wheatgrass genotypes for tolerance to salinity stress during seed germination and seedling growth stage*. *HortScience* 52: 1125–1134.
- Sheikh Mohamadi M.H., Etemadi N., Nikbakht A., Farajpour M., Arab M., Majidi M.M. (2018) *Wheatgrass germination and seedling growth under osmotic stress*. *Agronomy J.* 110: 572–585.
- Sheikh Beig Goharrizi M., Dejahang A., Tohidfar M., Izadi Darbandi A., Carrillo N.J., Hajirezaei M., Vahdati K. (2016) *Agrobacterium mediated transformation of somatic embryos of Persian walnut using *fld* gene for osmotic stress tolerance*. *JAST* 18(2): 423–435.
- Shen H., Qian B., Chen W., Liu Z., Yang L., Zhang D., Liang W. (2010) *Immunogenicity of recombinant F4 (K88) fimbrial adhesin *FaeG* expressed in tobacco chloroplast*. *Acta Biochim. Biophys. Sin.* 42: 558–567.
- Soria-Guerra R.E., Alpuche-Solis A.G., Rosales-Mendoza S., Moreno-Fierros L., Bendik E.M., Martínez-González L., Korban S.S. (2009) *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: 1293–1302.
- Svab Z., Hajdukiewicz P., Maliga P. (1990) *Stable transformation of plastids in higher plants*. *Proc. Natl Acad. Sci. USA* 87: 8526–8530.
- Thompson J.P., Abdolahi A., Noyes K. (2013) *Modelling the cost effectiveness of disease-modifying treatments for multiple sclerosis*. *Pharmacoeconomics* 31: 455–469.
- van Eerde A., Gottschamel J., Bock R., Hansen K.E.A., Munang'andu H.M., Daniell H., Liu Clarke J. (2019) *Production of tetravalent dengue virus envelope protein domain III based antigens in lettuce chloroplasts and immunologic analysis for future oral vaccine development*. *Plant Biotechnol. J.* 17: 1408–1417.
- Watson J., Koya V., Leppla S.H., Daniell H. (2004) *Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop*. *Vaccine* 22: 4374–4384.
- Wirth S., Calamante G., Mentaberry A., Bussmann L., Lattanzi M., Barañao L., Bravo-Almonacid F. (2004) *Expression of active human epidermal growth factor (*hEGF*) in tobacco plants by integrative and non-integrative systems*. *Mol. Breed.* 13: 23–35.
- Wood L. Access (2020) *Global interferons market outlook 2020-2030 – outbreak of COVID-19 has driven demand due to ongoing trials on interferons as potential therapeutic options*. www.businesswire.com 2020.

- Younessi-Hamzekhanlu M., Abdipour M., Dejahang A., Sabzi-Nojadeh M., Amani M. (2021) *Herbals used in Western Iran as food and for health treatments*. [in:] *Biodiversity, conservation and sustainability in Asia*. vol. 1: *Prospects and challenges in West Asia and Caucasus*. Ed. Ozturk M. Altay V., Efe R. Springer International Publishing: 547–599.
- Younessi-Hamzekhanlu M., Sanjari S., Dejahang A., Karkaj E.S., Nojadeh M.S., Gönenç T.M., Ozturk M. (2020) *Evaluation of essential oil from different *Artemisia fragrans* Willd. populations: chemical composition, antioxidant, and antibacterial activity*. *J. Essent. Oil-Bear. Plants* 23: 1218–1236.