High level expression and chloroplast targeting of human calcitonin (hCT) in transgenic tobacco plants

Article · November 2011

CITATIONS
2

READS
114

5 authors, including:

Farzaneh Kordbacheh
Dana-Farber Cancer Institute
25 PUBLICATIONS **321** CITATIONS
SEE PROFILE

Hamideh Ofoghi
Iranian Research Organization for Science and Technology
57 PUBLICATIONS **186** CITATIONS
SEE PROFILE

Ali Akbarzadeh
Gene Transfer Pioneers
20 PUBLICATIONS **69** CITATIONS
SEE PROFILE

Mahya Jafari
National Institute of Genetic Engineering and Biotechnology
25 PUBLICATIONS **55** CITATIONS
SEE PROFILE

Transgenic Plant Journal ©2011 Global Science Books

High Level Expression and Chloroplast Targeting of Human Calcitonin (hCT) in Transgenic Tobacco Plants

Farzaneh Kordbacheh[1,3] • Hamideh Ofoghi[2] • Ali Akbarzadeh[1,3] • Mahyat Jafari[1] • Ali-Hatef Salmanian[1*]

[1] Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[2] Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran
[3] Department of Cellular and Molecular Biology, Khatam Institute of Higher Education, Tehran, Iran

Corresponding author: * **Salman@nigeb.ac.ir**

ABSTRACT

Human calcitonin (hCT) is a 32-amino acid hormone which plays important roles in the regulation of calcium metabolism, treatment of osteoporosis, inhibition of osteoclastic activity and Paget bone disease. Recombinant hCT has been previously produced at low concentrations in plant-based systems with biological activity. To investigate if hCT could be effectively produced and accumulate at high concentrations in a plant system, we established transgenic tobacco plant lines expressing an optimized synthetic gene construct. Several strategies were applied to increase production levels including a strong over expression promoter, modified codon usage and organelle targeting. An hCT expression construct with tobacco codon preference was synthesized and attached to a chloroplastic signal sequence from the Rubisco small subunit gene of *Brassica napus* L. The construct was created by designing specific primers, using Polymerase Chain Reaction (PCR) and overlapping extension methods. *Agrobacterium*-mediated transformation was used to establish transgenic tobacco plants. The transgenic plants were analyzed by molecular methods and hCT concentration was determined by quantitative EASIA. hCT was expressed in the cytoplasm and accumulated in the chloroplast with a 26% efficiency. This is the first report describing expression and chloroplast targeting of hCT in transgenic plants.

Keywords: bio-pharmaceutical, plastid accumulation
Abbreviations: BnTP, *Brassica napus* L. chloroplast transient peptide; **CTAB**, cetyltrimethyl ammonium bromide; **DIG**, digoxigenin; **EASIA**, enzyme amplified sensitivity immunoassay; **GUS**, β-glucuronidase; **hCT**, human calcitonin; **HRP**, horseradish peroxidase; **RT-PCR**, reverse transcription PCR; **Rubisco**, ribulose 1-5 bisphosphate carboxylase oxygenase; **SOE**, splice overlapping extension; **SPP**, stromal processing peptidase; **TP**, transient peptide

INTRODUCTION

Calcitonin (CT) is a conserved 32-amino acid peptide hormone produced by specialized C-parafullicular cells of the thyroid gland in mammals or by cells of ultimo-bronchial glands in fish (Wellis *et al*. 1985). Some structural elements are essential for biological activity of this peptide such as N-terminal Disulfide Bridge (Cys1-Cys7), amphipatic α-helix and C-terminal proline amidation (Merli *et al*. 1996).

The main function of CT is the physiological regulation of calcium levels in the blood. Due to this action, CT plays an important role in the treatment of human disease. Calcitonin activity is not species-specific, but the prolonged application of animal-derived calcitonin leads to a gradual decrease or loss of activity associated with immunological reactions. An increase in therapeutic demand and the high cost of synthetic peptide production lead to the biotechnological production of recombinant hCT (Hong *et al*. 2003). Early research focused on chemical synthesis of the hCT gene and expression in *E. coli* (Ivanov *et al*. 1987) and *S. cerevisiae* (Mironova *et al*. 1991). Unfortunately, these studies showed low levels of hCT gene expression in both systems. Moreover, biologically active hCT requires amidation at the C-terminus and resulted in a search for other eukaryotic expression systems (Merli *et al*. 1996).

The majority of therapeutic proteins produced today are made in bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) or mammalian cell culture (Walsh 2003, 2006; Demain and Vaishnav 2009). Other production systems under development include the yeast *Pichia pastoris*, insect cell culture and transgenic animals and plants. In general, transgenic plants offer several advantages over other recombinant protein production platforms (Rasala *et al*. 2010). Plant systems provide many of the post translation modifications required for the expression of functional mammalian proteins, including glycosylation and amidation. Recombinant hCT with biological activity has been previously reported in *Solanum tuberosum* (Ofoghi *et al*. 2000), but the low yield of the recombinant peptide limits production in this system.

To date, many commercially important recombinant proteins have been successfully expressed in plants. However, a high level of recombinant proteins accumulation has always been a critical factor. A possible approach for increasing recombinant protein production in transgenic plants is to localize proteins at different organelles (Hyunjong *et al*. 2006).

The chloroplast organelle has an extensive transportation system. It has been shown that a soluble protein can be accumulated at high levels when it is targeted to chloroplasts. Most of the nucleus-encoded chloroplast proteins are synthesized in the cytosol as precursors with an N-terminal targeting signal called the transit peptide, which functions as the ticket for entry into chloroplasts (Li and Chiu 2010). The nuclear encoded small subunit of Ribulose 1-5 Bisphosphate Carboxylase Oxygenase (Rubisco) is well characterised and contains a signal sequence that targets the protein to chloroplast (Kim *et al*. 2009). The tobacco plant is an established model system for the production of recombinant pharmaceutical proteins (Fischer and Schillberg 2004).

Received: 6 February, 2010. Accepted: 29 September, 2010.

Original Research Paper

Table 1 The sequences of used primers. CalRs has *Sac*I (underlined) and BNTPFx has *Xba*I sites (underlined) at the 5′ends.

Name of primers	Sequence of primers
BNCalF	5′-GGAGGAAGAGTTAACTCGTGTGGGAATCTGAGTAC- 3′
CalRs	5′-GAGCTCTTAAGGTGCTCCAACCCC- 3′
BNTPFx	5′-TCTAGAATGGCTTACTCTATGCTCT- 3′
CalBNR	5′-GTACTCAGATTCCCACACGAGTTAACTCTTCCTCC- 3′

To our knowledge, this research is the first report describing high-level nuclear expression and chloroplast accumulation of recombinant hCT in tobacco plant.

MATERIALS AND METHODS

Construction of chloroplast-targeted synthetic hCT expression vector

A synthetic hCT (114 bp) (Accession number EU 523227) with tobacco codon preference was supplied on the pPCRScript plasmid and was amplified with gene-specific BNCalF forward and CalRs reverse primers (**Table 1**). The chloroplast transient peptide sequence (169 bp) isolated from the Rubisco small subunit of *Brassica napus* L. (Noodije *et al*. 2007) was amplified from the pUC18 plasmid using forward BNTPFx and CalBNR reverse primers (**Table 1**). To avoid non-desirable nucleic acid substitutions, all the intermediate fragments were amplified with *pfu* polymerase (Fermentas) enzyme. In order to attach *B. napus* chloroplast transient peptide (BnTP) to hCT, the two PCR products were assembled in a two-step Splice Overlapping Extension (SOE) PCR using a High fidelity PCR Enzyme Mix (Fermentas). The TP-hCT amplicon was then cloned into a T/A cloning vector (pTZ57R/T, Fermentas) and confirmed by sequencing in both directions with the standard M13 forward and reverse primers (accession number EU 533949). The pTZ57R/T vector was digested with *Xba*I and *Sac*I (Fermentas) and TP-hCT was subcloned into the binary vector pBI121 (Clontech) replacing the β-glucuronidase (GUS) reporter gene.

The recombinant pBI121 plasmid was confirmed using restriction enzyme digestion and PCR amplification. The pBI121 expression cassette was comprised of a CaMV 35S promoter controlling the TP-hCT coding sequence and a NOS polyadenylation signal. The binary pBI121 recombinant plasmid was used with *Agrobacterium* to transform tobacco plants.

Transformation of tobacco plants

Recombinant binary vector pBI121-TP-hCT was introduced into competent *Agrobacterium-tumefaciens* LBA4404 cells using the standard freeze and thaw method (Hofgen and Willmitzer 1988). A sterile *Nicotiana tabacum* 'Samson' cultivar was transformed by the standard leaf disk method (Gallois and Marinho 1995). Elongated shoots (3-5 cm in height) were rooted in MS-agar selective medium (Murashige and Skoog 1962) containing 100 mg/l Kanamycin (Fluka, China) in the absence of any plant hormones. The rooted plants were transferred to soil, after 4 weeks, mature leaves from below the apical meristem were harvested and used for molecular analyses. The plants were grown in a misting chamber (80% relative humidity, 16-h photoperiod and light intensity of 60-80 $\mu Em^{-2} s^{-1}$) for 2-3 weeks. Plants were then transferred to the greenhouse conditions at 25 ± 2°C with a 16 h light and 8 h dark photoperiod and allowed to flower and set seed.

Molecular analyses of transgenic plants

1. PCR and RT- PCR analyses

Two hundred mg of leaf tissues from mature transgenic plants were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted using the standard cetyltrimethyl ammonium bromide (CTAB) method (Sambrook and Russell 2001). PCR was performed using ~100 ng of genomic DNA and BNTPFx and CalRs primers for TP-hCT. The thermal program: 95°C for 5 min as initial denaturation, followed by 30 cycles of denaturation (95°C for 30 sec), annealing (50°C for 30 sec) and extension (72°C for 30 sec) and additional 10 min at 72°C for final extension was applied. Amplified products were separated on 2% agarose gel and visualized by ethidium bromide staining. In the RT-PCR experiment, Total RNA was isolated from fresh leaves of transgenic and control tobacco plants by RNX^{TM}-RNA Extraction Solution (Cinnagene, Tehran, Iran) according to the manufacture's protocol. To prepare cDNA, 2 µg of total RNA was reverse transcribed using CalRs as the reverse primer and RT kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse Transcription PCR (RT-PCR) was performed in a total reaction volume of 25 µl, containing 5 µl of cDNA and the BNTPFx and CalRs primers using the thermal program as described above.

2. Southern blot analysis

Two samples of genomic DNA (100 µg each) from leaf tissue were digested with *Xba*I and *Hind*III, electrophoretically separated on a 1% agarose gel and transferred to a Hybond N+ membrane (Roche Applied Science, Germany) for overnight. A Digoxigenin (DIG)-labeled-hCT probe was prepared using a PCR Probe Synthesis Kit (Roche) following the manufacturer's instructions. After drying the membrane at 110°C for 1 hr, it was pre-hybridized for 1 hr and then hybridized against DIG-labeled hCT probe at 65°C overnight. After treatment with AP-conjugated antibody, the hybridized probe was detected using NBT/BCIP color substrate.

Chloroplast isolation

Leaves from transgenic and control plants were incubated in direct lamp light (intensity 60-80 $\mu Em^{-2} s^{-1}$) for 1 hr. Two hundred mg of leaves were homogenized in 8 ml of basic buffer (0.33 M sorbitol, 0.01 M KCl, 0.001 M EDTA, 0.5 M HEPES)(Merck, Germany). Then 92 ml of 0.33 M sorbitol solution was added as an isolation solution. Crushed tissues were filtered through double layered cheesecloth into fresh ice-chilled tubes and centrifuged at 3000 rpm, for 4 min at 4°C to pellet the chloroplasts. The green pellet containing chloroplasts was resuspended in 20 ml of isolation solution and centrifuged (3000 rpm/4 min/4°C). The chloroplasts were then resuspended in 1 ml of suspension solution (0.33 M sorbitol, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$) (Merck, Germany) and stored at 4°C.

Protein extraction from whole leaf and isolated chloroplasts

Two hundred mg of fresh leaf tissue was frozen in liquid nitrogen and homogenized in ice-chilled protein extraction buffer (50 mM HCl, 1 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 2% β-mercaptoethanol) (Merck, Germany). The solutions were then centrifuged (13000 rpm/15 min/4°C) and the supernatants were stored at -20°C for further analyses. For protein extraction from intact chloroplasts, the samples were homogenized in suspension solution (above) supplemented with 1 mM PMSF and 50 mM EDTA to inhibit protease activity. The chloroplasts were disrupted by Silent Crusher (Heidolph, Schwabach, Germany) at maximum speed for 20 min on ice. After centrifugation (13000 rpm, for 10 min at 4°C), the clear supernatant was stored at -20°C for further analyses

hCT peptide detection by quantitative enzyme amplified sensitivity immunoassay (EASIA)

The concentration of total protein from whole leaves and isolated chloroplasts were analyzed by the Bradford assay (Bradford 1976). The amplified sensitivity immunoassay (EASIA) test was performed on extracted protein samples using the Bio Source CT-

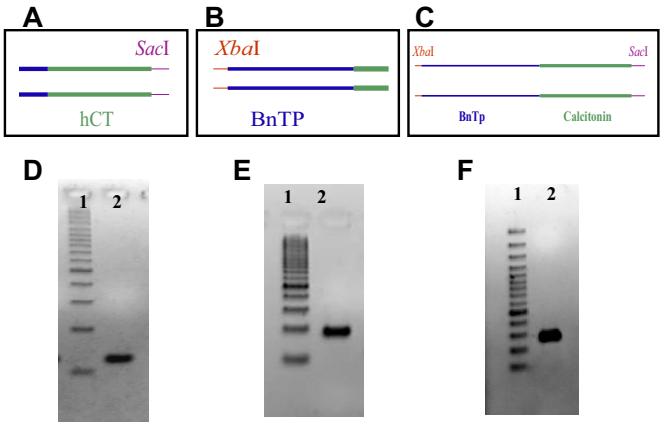

Fig. 1 Attachment of BnTP to hCT by SOE-PCR. (**A**) Schematic amplified hCT with 15 bp of BnTP at its 5′ end and *Sac*I site at 3′ end. (**B**) Schematic amplified BnTP with 15 bp of BnTP at 3′ end and *Xba*I site at 5′ end. (**C**) Schematic attachment of 2 amplified genes with *Xba*I site at 5′ and *Sac*I site at 3′ ends. (**D**) Line 2 is amplified hCT by BNCalF and CalRs primers (~115 bp). (**E**) Line 2 is amplified BnTP by BNTPFx and CalBNR primers (~ 175 bp). (**F**) Line 2 is attachment of BnTP and hCT by SOE-PCR (~270 bp). In every picture line 1 is 100 bp DNA ladders.

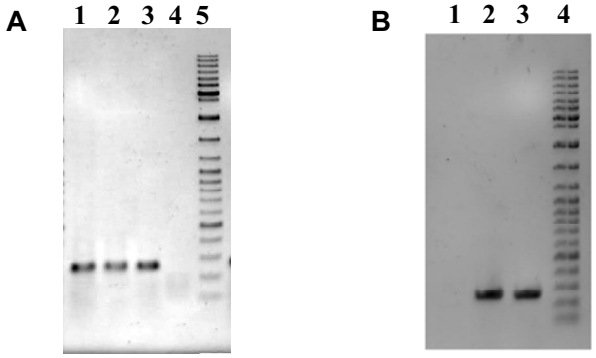

Fig. 2 PCR and RT-PCR analyses on transgenic plants. (**A**) Amplification of TP-hCT (~270 bp) in transgenic plants, lines 1 and 2 are amplified fragments from transgenic plants, line 3 is positive control and line 4 is wild type plant. (**B**) RT-PCR assay in transgenic plants, line 1 is wild type plant, line 2 is positive control and line 3 is RT-PCR product from a transgenic plant. The last line in both figures is 100 bp ladders.

U.S-EASIA Kit (BioSource Europe S.A., Belgium) according to manufacturer's instructions. In this method, calibrators and samples react with the capture monoclonal antibody (MAb 1) coated on microtiter well and a monoclonal antibody (MAb 2) labeled with horseradish peroxidase (HRP). After the formation of a sandwich (coated MAb 1 – human CT – MAb 2 – HRP), bound enzyme-labeled antibody was measured through a chromogenic reaction at 450-630 nm (T80 Double Beam UV/VIS Spectrophotometer).

RESULTS

Construction of TP-hCT expression vector

The amplification of the intermediate fragments and fusion of TP and hCT by SOE-PCR are shown in **Fig. 1**.

PCR screening of transformants and RT-PCR analysis

In order to confirm the integration of the TP-hCT construct into the plant genome and its transcription, PCR and RT-PCR techniques were performed, respectively. The pBI121-TP-hCT plasmid was used as a positive control and the genomic DNA from the transformed plants with a transformation vector lacking the TP-hCT gene served as a negative control (**Fig. 2A**).

The RT-PCR assay showed a single band of approximately 270 bp (corresponding to the size of the TP-hCT gene) in all transgenic plants; such a band was not seen in the RT-PCR products of negative control plants (**Fig. 2B**).

Southern blot analysis of transformants

In order to determine the copy number of T-DNA loci in transgenic plants, a transgenic line was analyzed by southern blotting. A transgenic line which was transformed with pBI121 vector was used as a negative control. Plant genomic DNA was separately digested with *Xba*I and *Hind*III and each was hybridised with a ~270 bp probe that targets the transient peptide and human Calcitonin gene sequences (**Fig. 3A**). The results from both digestion and hybridisations revealed that the transgenic line carried three copies of the TP-hCT construct. As there is a unique *Xba*I and *Hind*III sites in the recombinant TP-hCT-T-DNA construct, the number of hybridised bands indicated the integration of at least three separate TP-hCT loci copies in the genome of transgenic tobacco lines (**Fig. 3B**).

Quantitative hCT peptide detection by EASIA

The total protein concentration and quantitative hCT peptide EASIA test results of whole leaves and isolated chloroplasts of transgenic plants are presented in **Table 2**. The rate of hCT accumulation in chloroplasts is ~26% of total expressed hCT peptide in tobacco plant cells.

DISCUSSION

Following the initial purification and sequencing of Human Calcitonin (hCT), the peptide hormone was demonstrated to have a therapeutic efficacy in the prevention and treatment of osteoporosis. Production of recombinant hCT has been attempted in both prokaryotic and eukaryotic expression systems with only limited success.

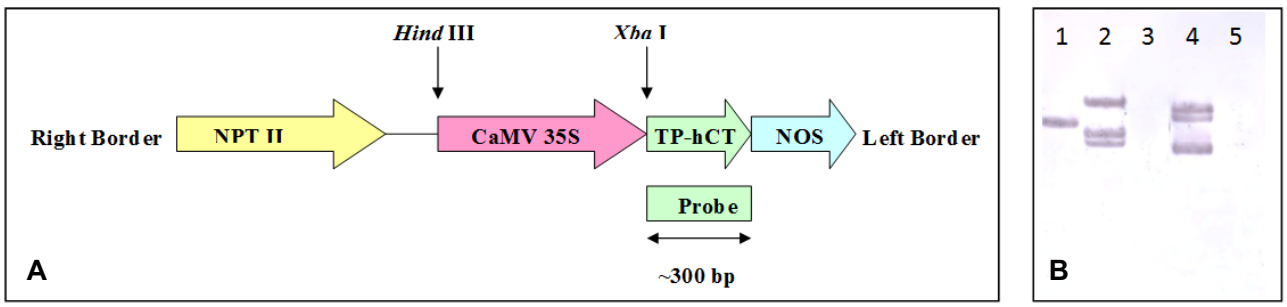

Fig. 3 Southern blot analysis of transgenes. (**A**) Schematic representation of recombinant binary vector T-DNA and position of *Xba*I and *Hind*III sites and the probe. (**B**) Southern blot analysis of *Xba*I and *Hind*III digested DNA isolated from T0 transgenic and control tobacco plants. Line 1 is pTZ57R/TP-hCT plasmid as positive control, lanes 2 and 4 are transgenic genomic plants digested with *Xba*I and *Hind*III, respectively; lines 3 and 5 are DNA digested with *Xba*I and *Hind*III from negative control plants.

Table 2 Quantitative analyses of hCT yields in transgenic tobacco plants using EASIA test data. The rate of chloroplast translocation was achieved by dividing the amount of cytoplasmic into chloroplastic hCT. For details see the text.

Location	Total soluble protein (ng mg^{-1})	EASIA test for hCT (ng mg^{-1})	hCT production rate (%)	hCT chloroplast translocation (%)
Cytoplasm	63,651	345 ± 5	0.54%	---
Chloroplast	20,136	91 ± 5	0.45%	26%

In general the recombinant proteins including pharmaceuticals can be expressed in bacteria, yeast, mammalian cell cultures and in transgenic animals or plants. Each expression system has its own disadvantages. In prokaryotes, recombinant peptides are highly susceptible to degradation in the cytosol (Levy 2008) and lack of post-translational modifications, such as glycoslyation and amidation (Sandgathe *et al*. 2003). Amidation of the C-terminus is essential for the biological activity of hCT and occurs only in higher eukaryotic cells (Hong *et al*. 2003). Transgenic animals and mammalian cells carry the risk of contamination with human pathogens; while, proteins purified from plants should be free from toxins and viral agents that may be present in preparations from bacteria or mammalian cell culture (Horn *et al*. 2004; Rasala *et al*. 2010). Plant systems are able to perform many of the post-translational modifications required for functional mammalian proteins (Gormond and Faye 2004; Rasala *et al*. 2010). However, despite their potential for cost effective large scale production (Schillberg *et al*. 2005), often the yields of recombinant peptides and proteins are low in plant hosts (Korban 2002; Hyunjong *et al*. 2006). Nuclear expression of transgenes enables regulated and tissue-specific expression, as well as posttranslational modifications (Rasala *et al*. 2010). Protein targeting to organelles (De Virgilio *et al*. 2008), specifically the chloroplast (Garg *et al*. 2007) has become a standard approach to increase the level of recombinant proteins in transgenic plants.

The Rubisco small subunit contains a highly conserved processing and cleavage site in the transient peptide (TP) amino acid sequence that is essential for efficient translocation and cleavage by the stromal processing peptidase (SPP) (Robinson and Ellis 1984). In this work this transient peptide was used for translocation of hCT peptide from cytoplasm in to chloroplast.

The expression construct (TP-hCT) was created by fusing the transient peptide and synthetic hCT gene, utilising PCR overlapping extension as to avoid any unwanted nucleotide alterations. The amplicon was verified by automatic sequencing.

Production of recombinant hCT in *Solanum tuberosum* has been previously reported, but the yield of hCT in this transgenic plant was only ~0.02% of the total cellular soluble protein (Ofoghi *et al*. 2000). To overcome to this problem several reports have addressed the improvement of protein expression in transgenic plants via the modification of the gene with plant optimized codon usage and the chloroplast targeting (Kim *et al*. 2008). In order to increase the level of protein expression and accumulation, we employed a combination of these strategies. Firstly, the codons of the hCT gene were adjusted to the plant codon preference by chemical synthesis. The GC content of synthetic hCT gene was decreased from 51 to 44% which is more suitable for expression in plant hosts (Geyer *et al*. 2007). Secondly, the synthetic hCT gene was fused to the chloroplastic transient peptide sequence isolated from *B. napus* Rubisco small subunit to provide efficient translocation. Thirdly, adding the *Xba*I site (TCTAGA) at the beginning of the TP fragment provided an Adenine (A) at position -3, Guanidine (G) at position +4 and Cytosine (C) at position +5, which restored an efficient translation signal for plant expression (Lutcke *et al*. 1987).

Southern blot analysis identified three separate copies of the TP-hCT cassette were integrated into the genome of transgenic plant. Transcription of the TP-hCT gene was verified by RT-PCR and this data shows there is no any apparent silencing in the transcription level in transgenic tobacco plant.

The expression of the recombinant hCT peptide and its translocation into chloroplasts were investigated by EASIA using specific antibodies. The results of quantitative EASIA showed that hCT production in leaves is ~345 ng/mg of the total cell soluble protein (0.54%), a 27-fold increase over the previous report in *S. tuberosum* plant expression system (Ofoghi *et al*. 2000). The increase in recombinant hCT expression demonstrates a combinatorial approach to optimising the gene sequence and other control elements can have a significant impact on transgene translation and protein accumulation.

The original hCT gene has two regions (amino acids number 13-15 and 20-23) that contain a series of codons with mammalian coding preference. In the synthetic hCT gene, these two codon groups were altered to plant coding preference. Moreover, like other heterologous expression systems, production of small peptides such as hCT as a fusion protein (TP-hCT) can protect it from cytosolic degradation (Sandgathe *et al*. 2003).

Furthermore, *in silico* analyses of the mRNA secondary structure using RNAfold Web Server (Gruber *et al.* 2008), predicted synthetic TP-hCT to be more stable (lower ΔG) than native hCT (data not shown). The mRNA stability could increase the molecule half life and results more protein synthesis.

Quantitative EASIA showed that the rate of hCT accumulation in chloroplasts was ~91 ng/mg (0.14%) of total cell soluble protein and it means near 26% of total hCT production was accumulated in the chloroplast. The BioSource CT-U.S-EASIA Kit that is used for *in vitro* quantitative measurement of the human Calcitonin (hCT) in blood serum and plasma samples was used for analysis of recombinant hCT. Therefore, the antigenic region of recombinant hCT must be immunologically the same as hCT and suggests structural similarity to the native hCT peptide hormone. Various forms of hCT exist in the plasma: monomer, oxidized monomer, dimer, higher molecular weight forms and possibly a precursor of hCT (Assicot *et al*. 1993; Rong *et al*. 1996; Andreotti and Motta 2004). The BioSource CT-U.S-EASIA Kit antibody is specific for the hCT monomer, suggesting the transgenic plant can produce a monomer form of recombinant hCT. The biological function of hCT is depending on amidation of proline at the carboxyl terminus and this post translational modification should be investigated for recombinant hCT which produced in tobacco plant. To our knowledge, this is the first report of expression and accumulation of recombinant hCT in chloroplast of a transgenic model plant. This work could introduce a novel plant system with high yields of recombinant hCT suitable for therapeutic application.

ACKNOWLEDGEMENTS

This project has been received partial financial support from project no. 278 at National Institute of Genetic Engineering and Biotechnology (NIGEB), and EMRO-WHO grant received by Iranian Research Organization for Science and Technology (IROST), Tehran, Iran.

REFERENCES

Andreotti G, Motta A (2004) Modulating calcitonin fibrillogenesis: An antiparallel α-helical dimer inhibits fibrillation of salmon calcitonin. *Journal of Biological Chemistry* **279**, 6364-6370

Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C (1993) High serum procalcitonin concentrations in patients with sepsis and infection. *Lancet* **341**, 515-518

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248-254

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances* **27**, 297-306

De Virgilio M, De Marchis F, Bellucci M, Mainieri D, Rossi M, Benvenuto E, Arcioni S, Vitale A (2008) The human immunodeficiency virus antigen Nef forms protein bodies in leaves of transgenic tobacco when fused to zeolin. *Journal of Experimental Botany* **59**, 2815-2829

Fischer R, Schillberg S (2004) *Molecular Farming: Plant-Made Pharmaceuticals and Technical Proteins*, Wiley-VCH, Weinheim, Germany, 315 pp

Gallois P, Marinho P (1995) Leaf disk transformation using *Agrobacterium tumefaciens*-expression of heterologous genes in tobacco. *Methods in Molecular Biology (Clifton, N.J.)* **49**, 39-48

Garg R, Tolbert M, Oakes JL, Clemente TE, Bost KL, Piller KJ (2007) Chloroplast targeting of FanC, the major antigenic subunit of *Escherichia coli* K99 fimbriae, in transgenic soybean. *Plant Cell Reports* **26**, 1011-1023

Geyer BC, Fletcher SP, Griffin TA, Lopker MJ, Soreq H, Mor TS (2007) Translational control of recombinant human acetylcholinesterase accumulation in plants. *BMC Biotechnology* **7**, 27

Gomord V, Faye L (2004) Posttranslational modification of therapeutic proteins in plants. *Current Opinion in Plant Biology* **7**, 171-181

Gruber AR, Lorenz R, Bernhart SH, Neubock R, Hofacker IL (2008) The Vienna RNA Websuite. *Nucleic Acids Research* **36 (2)**, w70-w74

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Research* **16**, 9877

Hong B, Wu B, Li Y (2003) Production of C-terminal amidated recombinant salmon calcitonin in *Streptomyces lividans*. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* **110**, 113-123

Horn ME, Woodard SL, Howard JA (2004) Plant molecular farming: Systems and products. *Plant Cell Reports* **22**, 711-720

Hyunjong B, Lee DS, Hwang I (2006) Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells. *Journal of Experimental Botany* **57 (1)**, 161-169

Ivanov I, Tam J, Wishart P, Jay E (1987) Chemical synthesis and expression of the human calcitonin gene. *Gene* **59**, 223-230

Kim EH, Suh SC, Park BS, Shin KS, Kweon SJ, Han EJ, Park SH, Kim YS, Kim JK (2009) Chloroplast-targeted expression of synthetic cry1Ac in transgenic rice as an alternative strategy for increased pest protection. *Planta* **230**, 397-405

Kim TG, Baek MY, Lee EK, Kwon TH, Yang MS (2008) Expression of human growth hormone in transgenic rice cellsuspension culture. *Plant Cell Reports* **27**, 885-891

Korban SS (2002) Targeting and expression of antigenic proteins in transgenic plants for production of edible oral vaccines. *In Vitro Cellular and Developmental Biology - Plant* **38**, 231-236

Levy WP (2008) Focusing on task of reinventing peptide drugs. *Genetic Engineering and Biotechnology News* **28**, 13 pp

Li H, Chiu CC (2010) Protein transport into chloroplasts. *Annual Review of Plant Biology* **61**, 157-180

Lutcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. *The EMBO Journal* **6**, 43-48

Merli S, De Falco S, Verdoliva A, Tortora M, Villain M, Silvi P, Cassani G, Fassina G (1996) An expression system for the single-step production of recombinant human amidated calcitonin. *Protein Expression and Purification* **7**, 347-354

Mironova R, Philipova D, Marekov L, Venkov P, Ivanov I (1991) Secretion of oligomeric Val^{8}-human calcitonin by Saccharomyces cerevisiae. *FEMS Microbiology Letters* **83**, 23-28

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Noodije AA, Salmanian AH, Mousavi A (2007) Isolation of transit peptide from small subunit of Rubisco enzyme from rapeseed and its analysis in transgenic tobacco plants. *Pajouhesh & Sazandegi* **20 (75)**, 175-181

Ofoghi H, Moazami N, Domonsky NN, Ivanov I (2000) Cloning and expression of human calcitonin genes in transgenic potato plants. *Biotechnology Letters* **22**, 611-615

Rasala BA, Muto M, Lee PA, Jager M, Cardoso RMF, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP (2010) Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal* **8**, 719-733

Robinson C, Ellis RJ (1984) Transport of proteins into chloroplasts. Partial purification of a chloroplast protease involved in the processing of important precursor polypeptides. *European Journal of Biochemistry* **142**, 337-342

Rong H, Saaf M, Torring O, Sjostedt U, Bucht E (1996) Circulating monomer-like calcitonin in osteoporotic patients. *Osteoporosis International* **6**, 394-398

Sambrook J, Russell WD (2001) *Molecular Cloning, A Laboratory Manual* (3^{rd} Edn), Cold Spring Harbor Laboratory Press, New York, pp 12.1-12.114

Sandgathe A, Tippe D, Dilsen S, Meens J, Halfar M, Weuster-Botz D, Freudl R, Thommes J, Kula MR (2003) Production of a human calcitonin precursor with Staphylococcus carnosus: Secretory expression and single-step recovery by expanded bed adsorption. *Process Biochemistry* **38**, 1351-1363

Schillberg S, Twyman RM, Fischer R (2005) Opportunities for recombinant antigen and antibody expression in transgenic plants - Technology assessment. *Vaccine* **23**, 1764-1769

Walsh G (2006) Biopharmaceutical benchmarks 2006. *Nature Biotechnology* **24**, 769-776

Walsh G (2003) Biopharmaceutical benchmarks 2003. *Nature Biotechnology* **21**, 865-870

Wellis M, Howell SL, Taylor KW (1986) *The Biochemistry of the Polypeptide Hormones*, John Wiley & Sons, New York, 488 pp