Tissue specific expression of human calcitonin gene in potato tubers by an organ specific promoter

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Abstract
To increase the production level of heterologous proteins in plants, strategies such as choice of stronger promoters, optimization of codon usage and specific localization of foreign proteins are of major concern. Calcitonin (CT), a 32 amino acid polypeptide is a powerful and specific inhibitor of bone resorption and is used to treat several human diseases. Calcitonin activity is not species-specific which make it possible to produce in various animal sources, however, antibody formation in the prolonged application of animal CT leads to gradual decrease or loss of activity. That is why the long term treatment of human patients with CT requires homologous calcitonin. In this study, a human calcitonin (hCT) gene, driven by two different promoters (granule bound starch synthase I and Cauliflower mosaic virus 35S) was expressed in potato plants, using *Agrobacterium*-mediated transformation. Molecular analysis, including PCR, RT-PCR, Northern dot blot hybridizations showed that hCT could be successfully transcribed in transgenic potato plants. The immunoassay results showed that tissue specific expression in potato, led to almost five-fold more hCT accumulation when compared to the constitutively expression in all plant tissues.
Keywords: Human calcitonin; transgenic potato; tuber; expression

INTRODUCTION

Plants offer a safe and extremely cost-effective alternative to microbial and mammalian expression systems for biopharmaceuticals products. Different strategies to improve plant expression systems have rapidly resulted in increased yield and successful down-stream processing of plant-made pharmaceuticals (PMPs). Such promising results, along with the fast progress in glycan humanization and reduced heterogeneity of plant made therapeutic proteins have made plants among the major expression systems, particularly when large quantities of multimeric recombinant proteins are required (Lienard *et al*., 2007; Fischer and Schillberg, 2004). To take full advantage of plant systems for the production of recombinant proteins, including therapeutic antibodies, it is important to study the tissue-specific factors involved in intracellular protein accumulation and post-translational modifications (PTMs). One of the major advantages of transgenic plants over other production systems available for large-scale and low-cost production, such as *E. coli* or yeasts, is their ability to perform most PTMs required for therapeutic protein's bioactivity and pharmacokinetics (Gomord *et al*., 2005; Gomordand Faye 2004).

Calcitonin (CT) is a 32-amino acid linear polypeptide hormone that is produced in human thyroidal parafollicular cells (also known as C-cells) as well as in the ultimobranchial bodies (thyroid-like glands) of avian species (Chan *et al*., 1969). Calcitonin is being produced by numerous non-thyroidal sources, including thymus, jejunum, lung, urinary bladder (Russwurm *et al*., 2001) mammary (Bucht *et al*., 1986) and pituary glands (Ren *et al*., 2001). This hormone is associated with maintaining calcium homeostasis and plays an important role as a pharmaceutical in treatment of human diseases like hypercalcemia or osteoporosis. The CT activity is not species-specific, however, due to immunological reactions the prolonged application

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of animal calcitonin leads to a gradual decrease or loss of activity. 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 high production costs and the increasing therapeutic needs for human calcitonin put forward ideas to develop a biotechnological process for its recombinant production (Hong *et al*., 2003). Recombinant DNA technology triggered numerous studies dedicated to the chemical synthesis of the hCT gene and its expression in *E. coli* (Ivanov *et al*., 1987) and yeast (Mironova *et al*., 1991). These studies showed that the hCT gene was poorly expressed in both bacteria and yeast. Moreover, the biological activity of hCT requires amidation and led to a search for other hosts with emphasis on eukaryotic origin.

Although potato tubers have been used as a plant host for the production of recombinant human proteins and vaccines (Arntzen *et* al., 2005; Henderson 2005; Ofoghi *et al*., 2005; Kim *et al*., 2003; Farran *et al*., 2002; Parkand Cheong, 2002; Ofoghi *et al*., 2000), the low level of target protein expression is a major barrier to recombinant protein production. For instance, a study of transgenic potato plants expressing a human serum albumin gene revealed that recombinant albumin accumulated up to 0.2% of total soluble tuber protein (Farran *et al*., 2002). In addition, the steps involved in purification of target proteins are major cost factors in plant-based protein production (Arntzen *et al*., 2005; Kim *et al*., 2003). The potato tuber low hydrolytic profile, which facilitates protein stability, and localization of foreign proteins and availability of strong tissue specific promoters which can boost the production of heterologous proteins to much higher amounts (De Jaeger *et al*., 2002) in tubers were the main driving forces for the tuber specific expression of the hTC gene.

MATERIALS AND METHODS

Microorganisms, plasmids and gene source: In this study, *Escherichia coli* XL1-Blue (Strategene) and *Agrobacterium tumefaciens* PGV3850 (Molecular Genetics Institute, Moscow) were used. The plasmid pPCRScript (Stratagene, Germany) was used as a source of potato plant codon preference to optimize the synthetic hCT gene (Accession number EU 523227). The pGB121s (provided by prof. R.G. Visser, Laboratory of Plant Breeding, Wageningen University, The Netherlands), and pBin19 (provided by Prof. Piroozian, Institute of Molecular Genetics, Moscow) binary vector plasmids harboring granule bound starch synthase I (GBSSI) and cauliflower mosaic virus 35S (CaMV 35S) promoters, respectively were used to construct the expression cassettes for potato plant transformation.

Plant cultivar and growth: *Solanum tuberosum* cv Kardal was kindly provided by prof. R.G. Visser, Laboratory of Plant Breeding, Wageningen University, the Netherlands. The plants were grown on sterile MS-based media (Murashige and Skoog 1962) under 16 hours light and 8 hours dark photoperiod. The potato mini-tubers were initiated from sterile in vitro grown potato shoots with 5-6 fully expanded leaves. After rooting on MS media containing 6% sucrose and 1 mg/l 6-benzylaminopurine (BAP), the plants were transferred to a dark room and incubated at 4°C for 7-10 days. Subsequently, plants were grown in a well-shadowed room at 16-18° C for mini-tuber formation.

Construction of plant expression vectors: Potato codon usage synthetic hCT (accession number EU 523227) gene was excised from the source plasmid pPCRScript (Stratagene) as *Bam*HI-*Bam*HI fragment and ligated into the *Bam*HI site of corresponding vector. The Expression cassette containing the CaMV35S promoter, hCT gene and the nopaline synthase terminator was digested by *Hind*III and subsequently introduced into pBin19 plasmid to provide non-tissue specific expression in potato (Fig. 1A). For tissue specific expression, *a Bam*HI-*Bam*HI fragment of the hCT gene was recloned in the *Bam*HI site of pGB121s (Fig.1B). The accuracy of hCT gene orientation in both obtained expression cassettes were confirmed by restriction enzyme digestion of the constructs and PCR amplification with specific calcitonin forward (CalF, 5′-ACGGATCCAATGTGCGGTAATCTGAGTACTTGC-3′) and reverse (CalR, 5′-GAAGATCTTAAGGTGCTC-CAACCC-3′) primers, CaMV 35S promoter forward (pCaMVF, 5′-GGCGAACAGTTCATACAGAGTCT-3′) and NOS-terminator reverse (Nos-terR, 5′-TTCATATATTTAGCCCTGTTCA-3′) primers. Furthermore, to check the accuracy of expression vectors, both constructs were sequenced. The recombinant plasmids were used for *Agrobacterium* mediated potato plant transformation.

Potato plant transformation and regeneration: Recombinant pGB121s-hCT and pBin19-hCT plas-

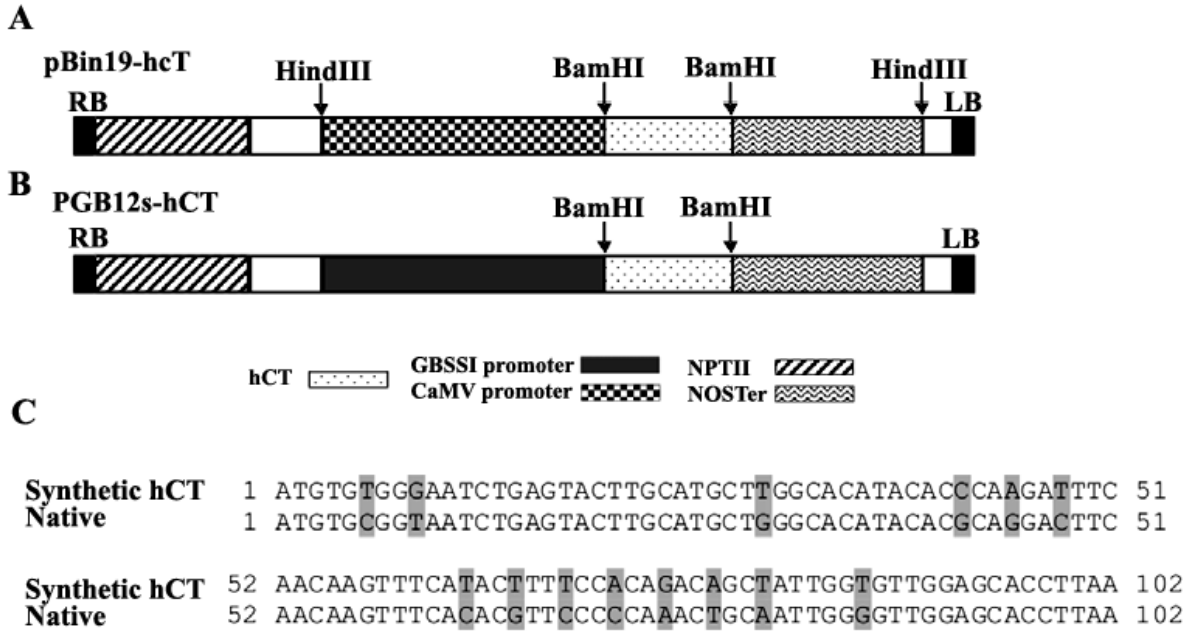

Figure 1. DNA constructs designed to express human calcitonin gene in potato plants. A: pBin19-hCT and B: pGB121s-hCT. RB and LB stand for right and left borders, respectively. C: The sequence alignment of synthetic and the native form of hCT are shown. Gray boxes indicate the position of substituted nucleotides in comparison to the native forms according to potato codon usage.

mids were introduced into competent *Agrobacterium tumefaciens* strain PGV3850 using the freeze and thaw standard method (An *et al*., 1988). The *in vitro* grown potato mini-tubers of 4-6 months old were cut into 1-2 mm slices and transformed by cocultivation with the *Agrobacterium* for 10 min carrying the corresponding plasmids (Ishida *et al.,* 1989). Tuber discs were subsequently transferred to plates containing MS medium with 100 µg kanamycin and 500 µg cefotaxime per mililiter. Plates were incubated in the dark for at least 2-3 days at 26°C for shoot regeneration. Regenerated shoots, 1.5-2cm long were cut, transferred to separate tubes containing the same medium and incubated at 26°C in a climate chamber. All transgenic plants were grown under controlled conditions for production of transgenic mini tubers which were used in further analysis.

Molecular analysis of transgenic plants

PCR and RT- PCR analyses: Two hundred milligram of various types of plant tissues from mature transgenic plants were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted using Cetyltrimethylammonium bromide (CTAB) method (Chen *et al*., 2008) PCR was performed by using approximately 100ng of genomic DNA and CalF and CalR primers using the following thermal program: 95ºC for 5 min as initial denaturation, followed by 30 cycles of denaturation (95ºC for 30 seconds), annealing (50ºC for 30 seconds) and extension (72ºC for 30 seconds) and additional 10 min at 72ºC as final extension. Amplified products were separated on 2% agarose gel and visualized by ethidium bromide staining.

Total RNA was prepared by RNX^{TM}-RNA Extraction kit (Cinnagene, Tehran, Iran) according to the manufacturer's protocol. To prepare cDNA, 2 µg of total RNA was reversely transcripted, using CalR as reverse primer and RT kit (Qiagen, Germany) according to the manufacturer's instruction. Reverse Transcription (RT) PCR reaction was performed in a total volume of 25 µl, containing 5 µl of cDNA using two specific CalF and CalR primers.

Northern Dot-blot analysis: In order to analyze transcription of hCT gene, total RNA from various types of plant tissue of transgenic potato plants was spotted on nitrocellulose (NC) membrane filters and hybridized with a specific DNA probe labeled with digoxigenin (DIG) using a commercial labeling and detection kit (Roche, applied science, Germany) according to manufacturer's instructions. Following drying at 110ºC for 1 hour, the membrane was pre-hybridized for 1 hour and then hybridized against DIG-labeled hCT probe at

65ºC overnight. After treatment with AP-conjugated antibody, the hybridized probe was detected using NBT/BCIP color substrate.

Protein Extraction: Green parts and mini-tubers of plant tissues frozen in liquid nitrogen were crushed into fine powder and homogenized in 2 volumes of ice-chilled protein extraction buffer (20 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% Triton X-100 and 2 mM phenyl methyl sulfoanylfloride (Mason *et al*., 1992). The solutions were then centrifuged 13000g/30 min/4ºC and the supernatants stored at -20ºC for further analyses. Tissue extracts were enriched in hCT by mixing with an equal volume of ice-cold 2 M acetic acid, incubated for 30 min on ice under gentle stirring and centrifuged at 13000 g for 20 min at 4ºC. The supernatant was decanted and used for analysis by EASIA (Ofoghi *et al*., 2005).

Quantitative Enzyme Amplified Sensitivity Immunoassay (EASIA) for hCT detection: To determine the concentration of proteins, the total protein contents of the whole plant and potato tubers were analyzed by Bradford assay (Bradford 1976). The EASIA test was repeated three times on extracted protein samples, using BioSource CT-U.S-EASIA Kit (BioSource Europe S.A., Belgium), following the manufacturer's instruction. In this method, calibrators and samples react with the captured monoclonal antibody (MAb 1) coated in a microtiter well and with 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 is measured through a chromogenic reaction. This reaction is stopped with the addition of stop solution and the optical absorbance of the samples was determined at 450-630 nm.

RESULTS

Identification of transgenic potato plants producing Hct: For each construct, at least 30 putative transgenic potato plants were generated. To confirm the successful incorporation of the hCT gene in the potato genome, PCR analyses were performed on all putative transformed plants, using hCT specific primers. The pBin19-hCT and pBG121s-hCT plasmids were used as positive controls and the genomic DNA from transformed plants with a transformation vector lacking the hCT gene served as negative control. Gel electrophoresis profiles revealed a band of 114 bp which corresponded well with the expected size of the gene of interest (Data not shown). No band was amplified in non transformed control plants. Furthermore, RT-PCR analyses on all transgenic plants showing the insertion of hCT gene from PCR analyses, using specific CalF and CalR primers, confirmed the efficient transcription of the gene of interest, whereas such a band was not found in the RT-PCR products of non transformed control plants (Fig. 2). Based on these experiments, transgenic potato plants number 7, 13 and 15 from pGB121s-hCT and transgenic number 1, 9 and 21 from pBin19-hCT were selected for further analyses. The untransformed control Kardal plants are referred to as KD-UT.

Northern dot-blot analysis: To further investigate the presence of the hCT gene transcripts in transgenic potato plants, Northern dot-blot hybridization analysis was used. Northern analyses on selected transformants showed the presence of hCT transcript in all three selected transformants of each construct, whereas no hCT mRNA transcript was detected in wild type

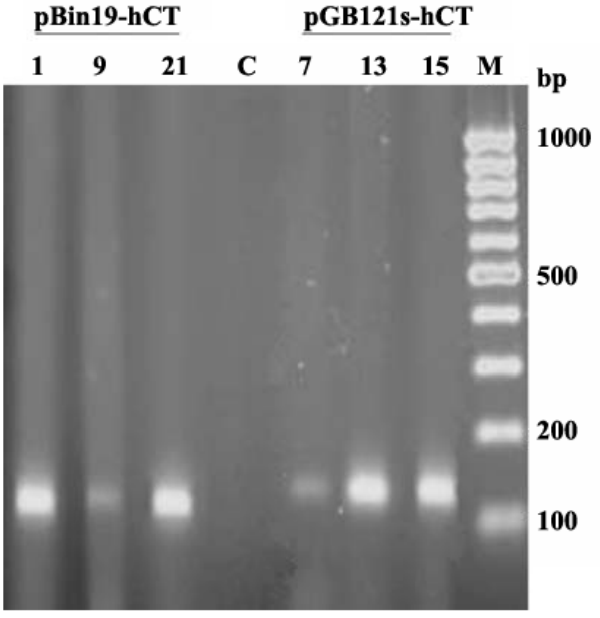

Figure 2. RT-PCR analyses on selected transgenic potato plants based on preliminary PCR. M shows the migration of 1kb DNA ladder. RNA was isolated from selected transgenic plant tissues, and RT-PCR was performed with specific primers that specifically amplified a DNA fragment of about 100 bp. C shows the untransformed Kardal potato plant. Total RNA were used as a templet for RT-PCR with specific hCT primers to confirm lack of DNA contamination (data not shown).

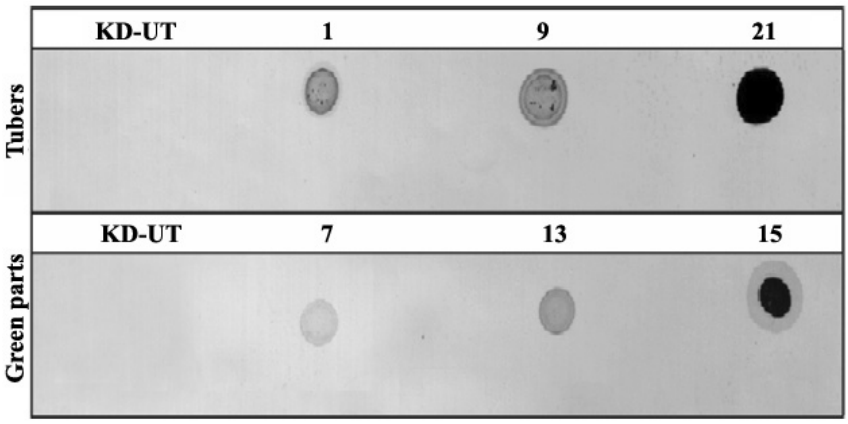

Figure 3. Dot-blot analysis of total RNA isolated from hCT transgenic and non transformed control plants. Total soluble extract from transgenic and non-transgenic potato plants were used in the blotting procedure. Alkaline phosphatase (AP) conjugate and NBT/BCIP color substrate were used to visualize the protein.

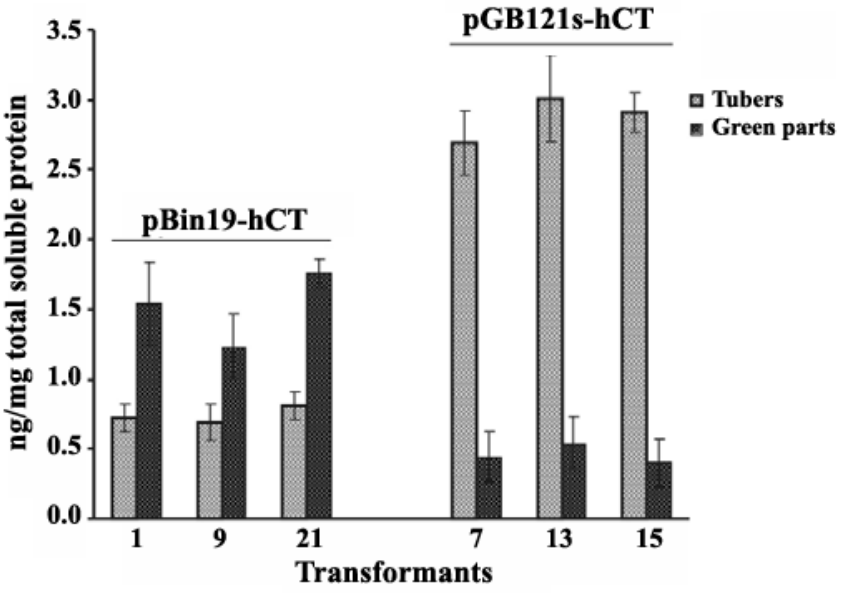

Figure 4. Quantitative analyses of hCT yield in various types of plant tissue of transgenic potato plants using EASIA test data. EASIA did not detect hCT in untransformed control plants. Data are the mean values for 3 measurements. Bars indicate the standard deviation from the mean values.

plants, as can be seen in the Figure 3. Moreover, the amount of hCT mRNA transcripts is much higher in tubers of transgenic plants expressing the gene in the tubers compared with those of constitutively expressing transgenic plants (Fig. 3).

Quantitative Enzyme Amplified Sensitivity Immunoassay (EASIA) of hCT: Total protein from various types of plant tissue of transgenic potato plants were subjected to EASIA analyses. The accumulation of hCT protein was quantified in four transformed plants (Fig. 4). Interestingly, the tissue specific expression of hCT in the tubers led to approximately about 5 fold higher hCT accumulation than constitutive expression of the gene. Furthermore, there seems to be a correlation between the amount of mRNA transcripts and calcitonin protein accumulation, as it can be seen from the Figure 4.

DISCUSSION

Plant genetic engineering offers the pharmaceutical industry exciting new opportunities to produce different proteins for therapeutic use. Due to the capability of plant cells for eukaryotic PTMs, transgenic plants have become attractive systems for production of human therapeutics. Despite the great potential for large scale and low cost production (Schillberg *et al*. ,2005), the level of expression in transgenic plants is still low. To date, different strategies like optimization of codon usage, using translational or transcriptional enhancer sequences (Ofoghi *et al*., 2005), protein targeting by signal peptides and expression of foreign gene under the control of tissue specific promoters (De Virgilio *et al*., 2008; Korban 2002) have become routine procedures to increase recombinant protein production and its accumulation in transgenic plants.

Since the discovery of hCT (Copp *et al*., 1962), production of hCT as a recombinant polypeptide in prokaryotic and eukaryotic systems has extensively been studied. Prokaryote systems such as *E. coli*, have their own disadvantages such as high risk of degradation (Levy, 2008) and lack of PTMs, including C-terminal amidation (Dilsen *et al*., 2000; Hong *et al*. ,2003). On the other hand, using higher eukaryotic hosts such as mammalian cells (Takahashi *et al*., 1997) or transgenic animals (McKee *et al*., 1998) to produce human therapeutics bear the risk of contamination with human pathogens or prions. In the present study, we try to increase the low yield of recombinant human calcitonin expression in transgenic potato plants. To achieve this aim and increase the expression and accumulation level, a combination of different strategies was used. Firstly, some codons of human calcitonin gene (amino acids number 13-15 and 20-23) were altered and it was chemically synthesized with plant codon preferences. By this method the GC content of synthetic hCT decreased from 51% to 44% which is more suitable for expression in plant hosts (Geyer *et al*., 2007). Secondly, the expression of synthetic hCT gene was driven by the tissue specific GBSSI promoter. Some studies suggested that the tissue specific expression in the plants compartment or organs will result in a much higher recombinant protein production as compared to constitutive expression. For instance production of a recombinant human granulocyte-macrophage colony stimulating factor (hGM-CSF) proved that using a tissue specific promoter could lead to 1,000-fold higher protein than the CaMV 35S promoter (Shin *et al*., 2003). Although our tissue expression did not result to such a massive expression, higher expression was achieved which is in line with the advantages of tuber expression.

The integration of hCT expression cassette in the potato genome resulted in the production of hCT in transgenic potato plants. The results of quantitative EASIA test showed that the amount of hCT production in potato green part extracts was about 1.8 ng/mg of the total soluble proteins (TSP), which was more than previously reported in plant expression systems. (Ofoghi *et al*., 2000) This amount of expression for recombinant hCT show that the combination of introduced changes to the gene sequence (Fig. 1C) and other control elements which could have impact on gene translation, appeared efficient. *In silico* analyses of synthetic hCT mRNA secondary structure showed that plant made hCT is more stable than native hCT one (data not shown). Nevertheless, the original hCT gene has two regions (amino acids number 13-15 and 20-23) which contain a series of codons, which are being used with low frequency in plant genome. In synthetic hCT gene, these two groups of codons altered to codons which are more familiar for plant translation system (Fig. 1C). Localization of hCT production in potato tubers led to 3.1 ng/mg total soluble proteins in comparison with only 0.68 ng/mg of total soluble protein when CaMV 35S promoter was deployed. These results showed that tissue specific expression in potato led to almost 5 fold higher hCT production than the constitutive 35 S promoter. However, our attempts to isolate the hCT from potato mini-tubers did not succeed. To achieve higher rate of hCT production in potato tubers, one may produce mature tuber with much higher yield potential.

The BioSource CT-U.S-EASIA Kit (Biosource, Europe) used for the *in vitro* quantitative measurement of hCT in serum and plasma samples suggested that the plant produced hCT protein must be structurally very similar to the native hormone. Among the various forms of CT in plasma, including a CT monomer, an oxidized monomer, a dimer, higher molecular weight forms, and possibly precursors of CT, this kit could detect only the monomer form and it can be postulated that the plant could preferably produce the monomer form of hCT. Nevertheless, the carboxyl terminal Proline-amidation which is critical for its biological activity in plant-based hCT should be investigated.

In conclusion, in the present study, the hCT gene modified on the basis of potato optimized codon usage and under the control of the granule bound starch synthase I promoter expression system, evidenced higher levels of

accumulated hCT expression in potato transgenic plants which is a consequence of the strength of the GBSSI promoter. To our knowledge, this is the first report of higher level expression and accumulation of hCT in tubers of transgenic potato plant. However, the amount of hCT production is still low and needs to be increased. Further molecular studies may shed light on an eukaryotic host system with acceptable level of hCT accumulation. Furtheremore, construction of other expression vectors carrying different tissue specific promoters and enhancing elements may result in higher accumulation of hCT level in potato tubers.

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