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COMPARING PATATIN CLASS I AND CAMV *35S* PROMOTERS IN EXPRESSION HUMAN CALCITONIN GENE IN POTATO (*SOLANUM TUBEROSUM* CVS. KARDAL A MARFONA)

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ABSTRACT

Calcitonin (CT), a 32 amino acid polypeptide hormone is a powerful and specific inhibitor of bone resorption and is used to treat several human diseases like hypercalcemia and osteoporosis. To date, many pharmaceutical proteins of mammalian origin have been synthesized in plants. To increase the production level of heterologous proteins in plants, strategies such as choice of stronger promoters and optimization of codon usage are of major concern. In this study, a human calcitonin (hCT) gene, driven by two different promoters (Patatin Class I and Cauliflower mosaic virus 35S) was expressed in two types of potato's cultivars (cvs) Kardal and Marfona plants, using Agrobacterium-mediated transformation. The transgenic plants were analyzed by molecular methods and hCT concentration was determined by quantitative EASIA. The results showed the localization of hCT production in Kardal potato tubers led to 0.7% of total soluble proteins whiles total soluble protein was 0.2% when CaMV 35S promoter was deployed, these results of Marfona when used Patatin Class I and Cauliflower mosaic virus 35S promoters were 0.3% of the total soluble protein and 0.1% of the total soluble protein in the respect. Data were analyzed by SPSS software using analytical statistics. There was significant difference in mean score of hCT production when two different promoters and two different cultivars were used. These results showed that organ specific expression in potato led to nearly 3 fold higher hCT accumulation than constitutive expression and Kardal cultivar expressed hCT about 2 times higher than Marfona cultivar.

Keywords: Human calcitonin, Transgenic potato, Bio-Pharmaceutical.

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Contribution/ Originality

The paper's primary contribution is finding that the expression of foreign gene under the control of organ specific promoter is more than constitutive promoter in transgenic potato. This study is one of very few studies which have investigated on the effect of two different potato plant cultivars for heterologous gene expression level.

1. 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.*, 1986). 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

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immunological reactions the prolonged application 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, however, that the hCT gene was poorly expressed in both bacteria and lower eukaryotes. The poor expression coupled with the fact that the biological activity of hCT requires amidation and led to a search for other hosts, particularly eukaryotes (Merli *et al*., 1996).

Other production systems under development include the yeast Pichia pastoris, insect cell culture and transgenic animals and plants. Recent studies have shown that use of plants as host has many practical, economic and safety advantages compared with more conventional systems. These include the low cost of production, rapid scalability, the absence of human pathogens and the ability to fold and assemble complex proteins accurately (Gomord *et al*., 2005); (Gomord and Faye, 2004); (Giddings, 2001); (Fischer and Emans, 2000).

Transgenic potato tubers as bioreactors offer advantages such as long-term storage tissue, abundant biomass, short growth cycle, high nutritional value and the high stability of recombinant proteins accumulated in the tubers during long period of storage (Kim *et al*., 2009); (Tremblay *et al*., 2011). Potato has been used in the last few years as a model system for the expression of bacterial, viral antigens, vaccines and recombinant human proteins (Shin *et al*., 2009); (Tacket, 2007); (Thanavala *et al*., 2005); (Arntzen *et al*., 2005); (Kok-Jacon *et al*., 2005); (Ma *et al*., 2005); (Ofoghi *et al*., 2005); (Joung *et al*., 2004); (Kim *et al*., 2003); (Farran *et al*., 2002); (Park and Cheong, 2002). 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 strong tissue and organ specific promoters in potato tuber can raise the production of heterologous proteins to much higher amounts (Tremblay *et al*., 2011); (Kim *et al*., 2009); (De Jaeger *et al*., 2002) .These advantages were the main driving forces for the tuber specific expression of the hTC gene.

In an attempt to increase the production of recombinant hCT, a coding region adapted to Solanum tuberosum codon usage were designed under the control of Cauliflower mosaic virus 35S promoter which is one of the strongest in potato leaves and quite strong in tubers. On the other hand, Patatin class I promoter for expression and accumulation of hCT gene in tubers were used. The expression cassette was made and ligated into the binary vector Bin19 and the resulting plasmid was introduced into Agrobacterium tumefaciens. These co cultivated with microtuber discs (obtained from tissue culture method) and transgenic plant regenerated from discs. After regeneration, tubers were obtained from tuberization MS medium and total plant protein extraction was used for determination of human calcitonin expression in transgenic potato by EASIA.

2. MATERIALS AND METHODS

2.1. Microorganisms, Plasmids and Gene Source

In this study, *Escherichia coli* DH5α (Strategene) was used in all molecular experiments and Agrobacterium tumefaciens LBA4404 was used for the plant transformation procedure. Bacteria were grown in Luria-Bertani (LB) medium containing, salt (NaCl) 10 g/L, Peptonase Casein 10 g/L and Yeast extract 5 g/L, at appropriate temperatures (37°C for *E.coli* and 28°C for *A.tumefaciens*). The plasmid PCR-Script (Stratagene, Germany) was used as a source of potato plant codon preference synthetic hCT gene (Accession number EU 523227).

The pPI plasmid, the pCaMV plasmid and pBin19 were used as a source for Patatin Class I promoter, cauliflower mosaic virus 35S promoter and subclonization of the expression cassette into potato plant in the respect.

2.2. Plant Cultivars and Growth

Solanum tuberosum cvs. Kardal and Marfona were grown on sterile MS-based media (Murashige and Skoog, 1962) containing MS salts with macro- and micronutrients, Sucrose 30 g/L, Agar 8 g/L, pH 5.7 under 16 hours light and 8 hours dark photoperiod and temperature regime 20° C light/18° C dark. Subsequently tuberization medium (Tovar *et al*., 1985) containing the MS basal medium supplemented with "1 mg/L 6-benzylaminopurine (BAP), 500 mg/L Chloro Cholin Chloride (CCC), 8% sucrose, pH: 5.7" was added to potato shoots with 5-6 fully expanded leaves. Then the plants were transferred to a dark room and incubated at 4°C for 7-10 days. Next, plants were grown in a well shadowed room at 16-18°C for micro-tuber formation.

2.3. Construction of Plant Expression Vectors

Potato codon usage synthetic hCT (accession number EU 523227) gene was excised from the source plasmid pPCR-Script (Stratagene) as *Bam*HI-*Bam*HI fragment, the digested products were separated on 1.5% low melting agarose gel and then was purified using the DNA extraction kit (BioNeer, South Korea). The fragment was directly ligated into the *Bam*HI site of corresponding vector. The expression cassette that contained the CaMV35S promoter, hCT gene and the nopaline synthase terminator was digested by *Hind*III and subsequently introduced into pBin19 plasmid for non-tissue specific expression in potato (named pBin19-hCT as shown in Fig.1-A). For tissue specific expression, the same restriction enzyme used to remove the CAT ORF from the pPI vector and *a Bam*HI-*Bam*HI fragment of hCT gene was recloned in the *Bam*HI site of pPI vector. For organ specific expression in potato tubers, The expression cassette containing the Patatin Class I promoter, hCT gene and the nopaline synthase terminator was digested by *Hind*III, introduced into pBin19 plasmid (named pPI-hCT as shown in Fig.1-B).

The accuracy of hCT gene orientation in both expression cassettes were confirmed by restriction enzyme digestion of the constructs and PCR amplification. The expression cassette containing the Patatin promoter, hCT and Nos terminator was digested by *Xba*I /*Sca*I (Fig. 2C) and other expression cassette digested by *Sca*I/*Eco*RI (Fig. 2D). Furthermore, both constructs were checked by PCR amplification with specific calcitonin forward (CalF, 5′ACGGATCCAATGTGCGGTAATCTGAGTACTTGC-3′) and reverse (CalR, 5′-GAAGATCTTAAGGTGCTCCAACCC- 3′) primers and NOS-terminator reverse (Nos-terR, 5′-TTCATATATTTAGCCCTGTTCA-3′) primers were synthesized by Cinnagen Company (Tehran, Iran). Expected 114 bp and 300bp fragments amplified by PCR from the pPI-hCT and pBin19-hCT when using CalF/ CalR and CalF/ NosR primers in the respect (Fig. 2E). The amplified products were migrated through the 1.5% agarose gel and were visualized by staining with ethidium bromide and photographed under ultraviolet light. The recombinant plasmids were used for Agrobacterium mediated potato plant transformation.

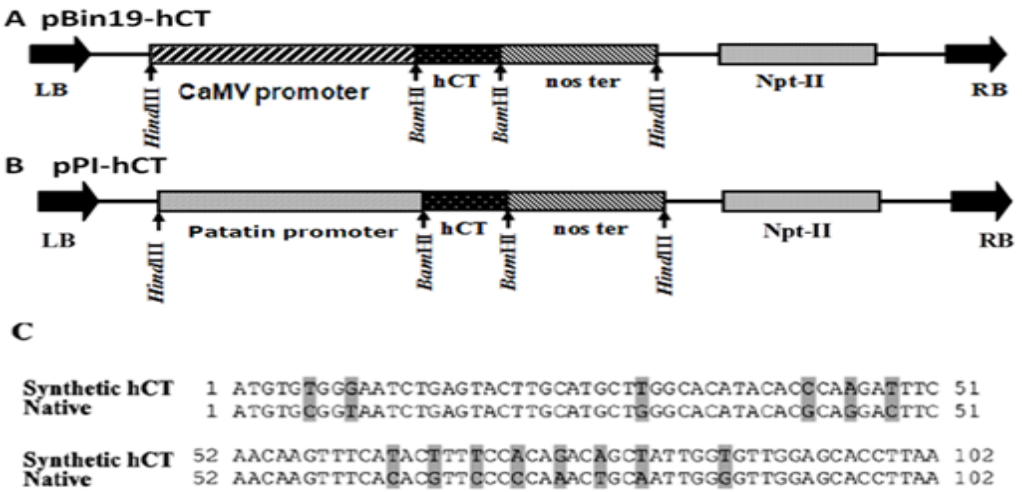

Fig-1. Schematic representation of Binary vector derived from pBIN19 used for the transformation of potato plants containing the hCT. A: hCT under the control of CaMV35S; B: hCT under the control of Patatin Class I promoter. RB and LB stand for right and left borders, respectively; C: The sequence alignment 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.
Source: We prepper this picture by ourself.

2.4. Potato Plant Transformation and Regeneration

Recombinant pPI-hCT and pBin19-hCT plasmids were introduced into competent *Agrobacterium* tumefaciens strain LBA4404 using the freeze and thaw standard method (An *et al.*, 1988). The in vitro grown potato micro-tubers of 4-6 months old, containing at least one bud, 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 MLS medium (Linsmaier and Skoog, 1965) containing MS macro salts, LS micro salts, 100 mg/l of inositol, 8 mg/l of thiamine-HCl, 8 mg/l of adenine sulfate, 2 mg/l of 6-benzylaminopurine (BAP), 1 mg/l of zeatin, 20 g/l of sucrose and 7.5 g/l of agar (Fermentase) without any antibiotics. The MLS medium was adjusted to pH 5.7 with 1 N HCl or NaOH before adding agar. The growth regulator BAP was added to the medium before autoclaving, but zeatin was filter sterilized and then added to the autoclaved medium. Plates were incubated in the dark for 24 hrs at 22°C then discs were rinsed by distilled water for *Agrobacterium* removal. They were then transferred to MLS selection medium containing 100 µg kanamycin and 500 µg cefotaxime per milliliter for shoot regeneration. Regenerated shoots, 1.5-2 cm in height were excised at the base individually and transferred to tubes containing the same medium and incubated under photo period condition (8 hrs dark/ 16hrs light at 18°C and 20°C in the respect) in a climate chamber. Shoots which apparently escaped from previous selection eventually died within 2-3 weeks on selective medium and the escapes mostly did not produce root. Two to three weeks after rooting, plantlets were transferred to MLS medium with only 100 µg kanamycin per milliliter. After elongated plants measuring 5-6 cm was added tuberization medium (Tovar *et al.*, 1985).

2.5. Molecular Analysis of Transgenic Plants

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 (Sambrook *et al.*, 1989) PCR was performed by using approximately 100ng of genomic DNA and CalF and CalR primers or CalF and NosR primers using the following thermal program: 94°C for 5 min; 30 cycles at 94°C for 1 min; 60°C for 1 min; 72°C for 1.5 min and a final extension at 72 °C for 5 min. Amplified products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Protein Extraction. Green parts and micro-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). Samples were centrifuged at 13000g. /30 min. /4°C and the supernatants stored at -20°C 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 4°C. 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.

2.6. Statistical Analysis

The SPSS software (version16.0.2) was used for statistical analysis. All quantitative data were analyzed using Student's t-test and are expressed as means ± standard deviation (SD). $p<0.05$ was considered significant.

3. RESULTS

3.1. Construction of pBin19-hCT and pPI-hCT Expression Vectors

Electrophoretic separation of DNA fragments are shown in Fig. 2.

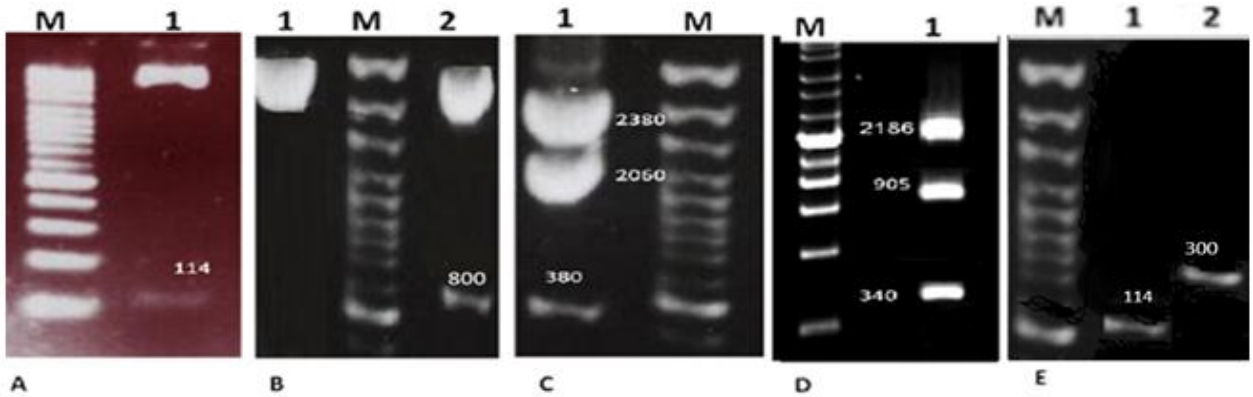

Fig-2.A- hCT extraction from pPCR-Script: M)100bp DNA size marker, 1)hCT gene (114 bp) excised from pPCR-Script with BamHI; **B-** Extraction of CAT fragment from pPI-CAT: 1) No digested pPI-CAT, M) middle range DNA size marker, 2) pPI-CAT digested with BamHI and separated CAT fragment (800 bp); **C-** Restriction map analysis confirming the accurate pPI-hCT binary vector construction: M) 100 bp DNA size marker,1) digestion of pPI-hCT with Xba /ScaI that released 381,2374 & 4511, bps fragments; **D-** Restriction map analysis confirming the accurate pBin19-hCT binary vector construction: M) DNA size marker 1 Kb, 1) digestion pBin19-hCT with ScaI/EcoRI that released 340,905 & 2186, bps fragments; **E-** PCR analysis confirming the accurate pPI-hCT binary vector construct: M) 100 bp DNA size marker, 1) CT fragment amplified by CalF & CalR ; 2) CT and Nos terminator fragment amplified by CalF & NosR. (Data not shown for pBin19-hCT)
Source: We prepper this picture by ourself. Photograph of our gel duc.

3.2. Transformation and Regeneration of Potato Plants

Genetic transformation of potato was efficient and resulted in enough numbers of transgenic plants. The transgenic plants were selected on regeneration medium containing 100 µg kanamycin per milliliter. All transgenic

plants appeared morphologically normal in comparison with non-transgenic plants. Transgenic micro-tubers were used in further analyses.

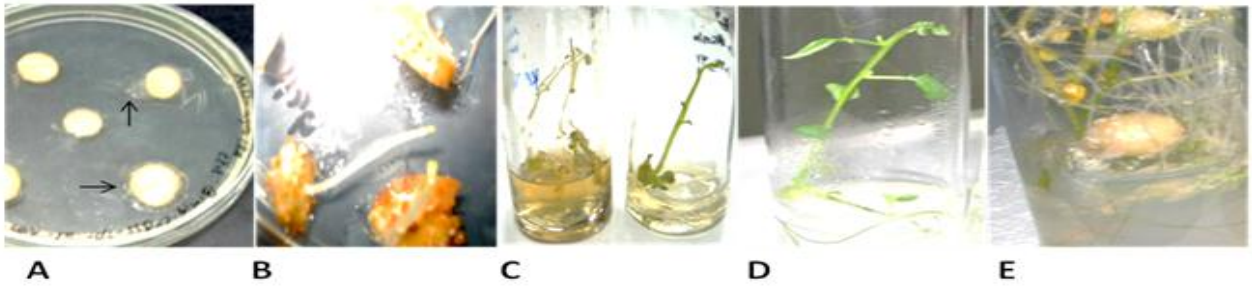

Fig-3. Transformation and regeneration of transgenic potato plants. A) Infected micro-tuber slices transformed with Agrobacterium; B) Regenerated shoots in growth medium containing antibiotics; C) Regenerated shoots transferred to new selective medium; D) Regenerated potato with well-developed roots and leaves; E) Tuber formation from transgenic potato.
Source: We prepper this picture by ourself. Photograph of our plants.

3.3. Identification of Transgenic Potato Plants Producing HCT

For each construct, at least 15 putative transgenic potato plants were generated. Transgene insertions in genomic DNA were verified by PCR screening, PCR analyses were performed on all putative transformed plants, using hCT specific primers. The pBin19-hCT and pPI-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. Based on this experiment, transgenic Kardal cultivar potato plants number 2, 8 and 11 from pPI-hCT and transgenic number 1, 9 and 10 from pBin19-hCT were selected for further analyses. Transgenic Marfona cultivar potato plants number 3, 5 and 8 from pPI-hCT and transgenic number 4, 6 and 9 from pBin19-hCT were selected for further analyses. The untransformed control Kardal and Marfona plants are referred to as KD-UT.

3.4. 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 three transformed plants (Fig. 4). Interestingly, the organ specific expression of hCT in the tubers led to approximately about 3 fold higher hCT accumulations than constitutive expression of the gene and Kardal cultivar expressed hCT about 2 times higher than Marfona cultivar.

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.
Source: We prepper this picture by ourself.

3.5. Statistical Results

Our results showed that, there were a significant difference between expression level under the control of Patatin class I promoter and Cauliflower Mosaic Virus 35S promoter. The mean of hCT expression was 1.86±0.05 ng/mg in Kardal tuber infected by pPI-hCT and 1.12±0.11 ng/mg in Kardal green parts infected by pBin-hCT, (p<0.05). Also, in the 2nd comparing, there was a significant difference in hCT expression between two cultivars when both of them were under the control of same promoter. The mean of hCT expression by pPI-hCT was 1.86±0.05 ng/mg in Kardal tuber and 0.98±0.09 ng/mg in Marfona tuber, (p<0.05).

4. DISCUSSION

Plants have been engineered to express foreign genes for agronomic traits since the early 1980s, but only more recently have been used as production vehicles for protein products (Karg and Kallio, 2009). However, despite plants 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).

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 organ accumulation (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. 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, for high-level transcription, one of the most important elements is the promoter, which is often derived from the 35S transcripts of the cauliflower mosaic virus (CaMV) (O'Dell *et al*., 1985); (Lawton, 1987). The CaMV 35S promoter is a strong constitutive promoter and popular choice in dicots. However, Promoters that allow expression of a transgene in a particular environment or tissue specific manner are more popular. For example, there are several advantages to the restriction of transgene expression to potato tubers using organ or tissue-specific promoters, such as granule bound starch synthase I and Patatin Class I promoters. The advantages of such promoters include the increased stability of the protein and the avoidance of protein accumulation in vegetative organs, so preventing toxicity to the host plant (Ma *et al*., 2005). For instance production of human serum albumin under the control of CaMV35S and Patatin B33 promoters proved that using an organ specific promoter could lead to 10-fold higher accumulated protein than the CaMV35S promoter (Sijmons *et al*., 1990); (Farran *et al*., 2002). Due to this subject, the expression of synthetic hCT gene was driven by the organ specific Patatin Class I promoter.

Thirdly, these constructs introduced to two different cultivars, owing to some findings showed that there are comparable differences between the conventional varieties that resulted as an unintended effect of genetic engineering techniques (Catchpole *et al*., 2005); (Mikschofsky *et al*., 2011).

Patatin Class I promoter was used for organ accumulation of hCT peptide in potato micro-tubers. The expression construct (pPI-hCT) was created by fusing promoter and synthetic hCT gene, subsequently the expression rate of this construct was surveyed in two types of potato cultivars and findings compared with other construct that it made of CaMV35S promoter. 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 under the control of CaMV35S was about 1.2 ng/mg of the total soluble proteins (TSP) for Kardal cultivar

and near to 0.6 ng/mg of TSP for Marfona cultivar, 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. Nevertheless, the original hCT gene has two regions (amino acids number 13-15 and 20-23) which contain a series of codons that 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 Kardal potato tubers led to 1.8 ng/mg total soluble proteins in comparison with only 0.7 ng/mg of total soluble protein when CaMV 35S promoter was deployed, for Marfona these results were 0.97 ng/mg of the total soluble protein and 0.34 ng/mg of the total soluble protein in the respect (Fig.4). So, the considerable success has achieved with the Patatin promoter in the potato micro-tuber of Kardal CV. These results showed that organ specific expression in potato led to almost 3 fold higher hCT production than the constitutive 35S promoter and Kardal cultivar has more ability in heterologous protein expression.

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 Patatin Class I promoter expression system. The results confirmed that the level of accumulated hCT expression in transgenic potato micro-tuber is a consequence of the strength of the pPI promoter and the effect of plant cultivar. Though the use of plants as hosts for the over-expression of foreign proteins is still a developing field and the amount of hCT production is still low and needs to be increased. Further, construction of other expression vectors carrying tissue specific promoters and enhancing elements and selecting of perfect cultivars may result in higher accumulation of hCT level in potato tubers.

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Contributors/Acknowledgement: All authors contributed equally to the conception and design of the study.

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