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Regeneration and *Agrobacterium*-mediated transformation of three potato cultivars (*Solanum tuberosum* cv. Desiree, Agria and Marfona) by human proinsulin gene

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Abstract

It is expected that the increasing outbreaks of diabetes mellitus along with introducing alternative of present insulin consumption methods, will result in increasing the demand of such a drug in future. Plant systems have high potential to produce safe, economical, inexpensive and large-scale biopharmaceuticals and potato is one of the most important bioreactors, globally. Most *Agrobacterium*-mediated transformation procedures referred to in the literatures have proven to be inefficient for the transformation of Agria and Marfona potato cultivars. In this study a novel one- step method was demonstrated for the transformation of the internodal explants of these cultivars. Using this method, human proinsulin gene was expressed in the transgenic potato plants. In this research, the DNA sequence of Immunoglobulin G binding protein taken from *Staphylococcus aureus* was infused to the human proinsulin gene and this construct was then transferred to the nuclear genome of potato via *Agrobacterium*-mediated transformation. Assessment of the transgenic plants was carried out in 3 levels of DNA, RNA and protein. Gel analysis of the PCR and RT-PCR products showed a single 500 bp band in the transgenic potato lines. SDS-PAGE analysis also showed a 17-18 kDa weight band, while this band was not shown in the non-transgenic ones. Leaf samples of transgenic potato had a positive reaction in ELISA with the human insulin antibody and dot blot assays were also positive.

Keywords: Bioreactor, Diabetes mellitus, Human proinsulin, Molecular farming, *Solanum tuberosum* L.
Abbreviations: DAB- 3, 3´- diaminobenzidine; ER- Endoplasmic Reticulum; GA_3- Gibberellic Acid; GFP- Green Fluorescent Protein; GUS- β- Glucuronidase; LSD- Least Significant Difference; NAA- α-Naphthalene Acetic Acid; TSP- Total Soluble Protein; ZR- Zeatin Riboside.

Introduction

In the late 1980s, recombinant DNA technology and protein production were used in plants and plant expression systems were able to produce more inexpensive and safer pharmaceutical proteins. Producing biopharmaceuticals as well as important proteins using plants is called molecular farming (Schillberg et al., 2002). Diabetes mellitus is a common disease resulting from a lack of insulin secretion or its malfunction. In industrial counties, insulin-dependent diabetes mellitus (Type I), is the third most common condition contributing to mortality after cardio-vascular disease and cancer (Barfoed, 1987). About 0.7% of the world's population is suffering from insulin-dependent diabetes mellitus (Winter et al., 2000). According to some assessments, the amount of insulin needed by diabetic people, considering the number of diabetics who are currently at the preliminary stages of the illness, the increasing number of diabetic people per year and the trend toward non-invading methods of insulin application such as insulin administration through the lungs, nose and mouth (with the absorption reduction of 5 to 20 times) it is predicted that insulin demand will increase in near future (Markley et al., 2006; Nykiforuk et al., 2006). Encountering such insulin demand, requires improving the modern, inexpensive and economic insulin production methods with high efficiency (Nykiforuk et al., 2006). Nowadays, commercial insulin production is done in *Escherichia coli* (Chan et al., 1981) and *Saccharomyces cerevisiae* (Thim et al., 1986). Although these commercial systems have successfully passed optimizing processes for the past two decades and have reached a production level of 5 tones of insulin per year, more of this hormone will be needed in the future. Various plants can be used to produce recombinant proteins but potato is a plant with several advantages over others. One of these is that potato produces transgenic mini-tubers in the completely controlled conditions of a growth chambers. Unlike many plants, potato does not need to be planted on a farm-scale. This reduces the possibility of gene escaping and cross pollination with the other plants of the same family resulting in increasing the safety of producing such a transgenic plant. Other advantages are as follows: independency to the growth season; having fewer phenol compounds in its tubers than in the green leaves; good storage capacity of the potato tuber; non-existence of pharmacologically active secondary metabolites in potato tubers; relative simplicity of potato genetic manipulation; non-sexual propagation of potato resulting in populations with the minimum risk of variation; the possibility of sterile transgenic plantlet multiplication using auxiliary buds on a commercial scale (During, 2005). Insulin

is a 51-amino acid polypeptide and has two separate chains A and B. The A chain has 21 amino acids and the B chain has 30 amino acids. These two chains bind together with two disulphide bonds. There is an internal molecular disulphide bond in the A chain. Insulin is produced in pancreatic beta-cells as preproinsulin. There is a part in preproinsulin called signal peptide, which conducts it to the endoplasmic reticulum. Proinsulin is produced via cleavage from the first 24 amino acids from the amino terminus of preproinsulin (Perler et al., 1980). Along with these two chains A and B, proinsulin has a peptide called C that separates in the secreting vesicle (Winter et al., 2000). Studies in humans have shown that proinsulin has a longer half-life than insulin (Stoll et al., 1970). *Solanum tuberosum* was among the first plants to be transformed. Transformation of this plant was first reported by Ooms et al. (1986) using *Agrobacterium rhizogenes*. Arakawa et al. (1998) reported that transgenic potato plants were produced and these plants synthesized human insulin at levels up to 0.05% of TSP. Transgenic potato tubers produced 0.1% of TSP as a pentameric CTB-insulin fusion. After processing and purification, the final net rate of insulin extracted from potato plants was 0.022% of TSP. In 2007, cholera toxin B-proinsulin fusion protein was expressed in lettuce and tobacco chloroplasts up to 2.5% and 16% TSP respectively (Ruhlman et al., 2007). For investigation the expression of insulin in *Arabidopsis thaliana* seeds, recombinant human precursor insulin (Des-B_{30}) fused with oleosin and interlinked with trypsin cleavable propeptide expressed 0.13% of total seed protein (Nykiforuk et al., 2006). Insulin has also been expressed in *Arabidopsis* by targeting a protein to oil bodies with an affinity tag, wherein the gene construct included Des-B_{30} insulin, trypsin, cleavable propeptide sequence, a KDEL (Lysin- aspartate-glutamate- leucine) endoplasmic reticulum (ER) retention signal peptide and an affinity tag for oleosin. In mentioned research, insulin was targeted to accumulate in ER and demonstrated that targeting insulin to ER and introducing modification in this gene construct has made it possible to achieved expression levels as 1.15% total seed protein (Markley et al., 2006). In this study, the DNA sequence of Immunoglobulin G binding protein taken from *Staphylococcus aureus* infused to the human proinsulin gene was transferred to the nuclear genome of potato via *Agrobacterium*- mediated transformation.

Results and Discussion

In statistical analysis for determination of the best concentrations of plant growth regulators, results of Anderson- Darling normality indicated that the data set was normal (data not shown). Based on Table 1, the treatments that the average differences of their shoot regeneration percentages were more than 14.39 and 19.71 were significant at 5% and 1% levels of probability, respectively. Because the average shoot regeneration percentage difference of 13.34 showed no significant difference between the two treatment combinations (Desiree-2 and Desiree-3), Desiree-2 treatment combination was used for Desiree shoot regeneration. According to this Table, Agria-2 and Marfona-3 treatment combinations were used for Agria and Marfona shoot regeneration respectively. Significant and non significant difference between other one-step treatment combinations is investigable by determination of the average difference of shoot regeneration percentages between two compared treatment combinations and then a comparison with the LSD at 5% and 1% level of probability (Table 1). It is necessary to mention that the first shoot regeneration was occurred within 21, 42 and 42 days from tissue culture initiation and reached to 90-100% within 10, 30 and 30 days from the first shoot regeneration in Desiree, Agria and Marfona cultivars respectively. To determinate the lethal dose of hygromycin, one-step regeneration media (containing 0, 5, 7.5, 10, 12.5, 15 and 20 mgl^{-1} of hygromycin) were used. The non-scratched internodal explants of the 3 to 4-week-old sterile plantlets of Desiree, Agria and Marfona cultivars were put in the culture media. Then, after shoot regeneration of all explants in the control medium (without hygromycin), the tolerance threshold of the assessed cultivars to hygromycin was thus determined. The results were 7.5, 12.5 and 10 mgl^{-1} for Desiree, Agria and Marfona cultivars, respectively. In order to indicate that transgene (protein A-proinsulin) integration in *Agrobacterium*, colony PCR method was used. A single 500 bp amplification product was produced that confirmed the existence of the mentioned gene in *Agrobacterium* (Fig 1). In this study, proinsulin gene transformation into potato plants was done by *Agrobacterium tumefaciens* LBA4404. The internodal explants of Desiree, Agria and Marfona cultivars inoculated with *Agrobacterium* harboring the target gene were put on a co-culture media having no hygromycin and cefotaxime antibiotics for 3 days in darkness and were then placed on the shoot regeneration media containing MS salts, B_5 vitamins, cefotaxime (500 mgl^{-1}) and hygromycin (7.5, 12.5 and 10 mgl^{-1} for Desiree, Agria and Marfona cultivars respectively).These inoculated explants were incubated at the temperature of 22±1˚C under conditions of 16 hours of daylight and 8 hours of darkness. After 4 to 5 weeks, some of the inoculated explants, in which the transgene had possibly become integrated in their cells as well as the hygromycin resistance gene, started to produce shoots. Simultaneously, shoot regeneration of all non-inoculated internodal explants occurred in the control medium (without hygromycin and cefotaxime) and shoot regeneration of non-inoculated internodal explants did not occur in the same medium without cefotaxime, but with hygromycin antibiotics (Fig 2). Afterwards, the regenerated shoots were transferred to the MS based culture medium containing 20 g l^{-1} sucrose and 500 mg l^{-1} cefotaxime. It is notable that the average regeneration percentage of Desiree cultivar internodal explants was 30.44±8.18 after transformation. After 3 to 4 weeks, green regenerated shoots were transferred to the MS culture media having 20 gl^{-1} sucrose, 500 mgl^{-1} cefotaxime and (7.5, 12.5 and 10 mgl^{-1}) hygromycin (for Desiree, Agria and Marfona cultivars, respectively) for further growth and root production. In this medium, the real transgenic plantlets stayed green and continued to grow. The achieved plants were first transferred to peat and perlite and then into the soil. These plants continued their growth in the green house at the temperature of 25˚C, in 16 hours of daylight and 8 hours of darkness. They were under specific cares during the growth procedure (Fig 2).

Analysis of regenerated plants

PCR and RT-PCR analysis

The extracted genomic DNA from potato plants were assessed qualitatively and quantitatively using 0.8% agarose gel and spectrophotometer. The quality and quantity of the extracted DNA samples were desirable for all analysis.

Several genomic DNA samples, extracted from the achieved plants of the selective media were used for a polymerase chain reaction using specific primers of the human proinsulin gene. Transformed plants displayed a 500 bp amplification product, which was missing in non-transformed control plants (Fig 3). In order to assess the gene transcription, RT-PCR was carried out using a positive and two negative controls (one had no template and another one had an extracted RNA as a template to show possible DNA contamination in extracted RNA). The result of RT-PCR showed a single 500 bp amplification product which was missing in non-transformed control plants and in negative controls (Fig 4).

Protein assays

Expression of the transgene in the regenerated plants was also verified by protein analysis. SDS-PAGE on the proteins extracted from the young leaves of transgenic potato plants showed a 17-18 kDa weight band but this band was not evident in non-transgenic plants. The weight of this protein was equal to its predicted size (Fig 5). ELISAs with random leaf samples showed the presence of human proinsulin protein in tested potato cultivars. Potato leaf samples had a positive reaction in ELISA with the human insulin antibody. According to the standard calibration curve of human insulin, the average amount of recombinant human proinsulin was estimated at 0.022% TSP. Selected positive samples were tested by dot blot and results showed them to be positive (Fig 6). Due to the importance and necessity of molecular farming, such technology is a priority of biotic research to facilitate inexpensive mass production of recombinant proteins. Generally, 39% of recombinant proteins have been produced by *Escherichia coli* as well as 35% by CHO cells, 15% by fermenters, 10% by mammalian cell culture systems and 1% by other bacteria and systems (Rader, 2008). Proteins accumulated in bacteria usually have inappropriate folds (Hockney, 1994; Lu et al., 1996). Also, the high cost of the mammalian cell culture medium, the possibility of being infected by viruses and perions and dependability on components of the culture medium were of those problems which are always along with mammalian cell culture (Griffiths and Electricwala, 1987). Recently, research on using plants to produce recombinant proteins has been regarded a lot due to its economical aspect, scale-accepting and safety of products (Hellwig et al., 2004). It is believed that plant bioreactors are more suitable than microbial systems duo to their cost, protein folding, storage and distribution. Other advantages of plant systems are the availability of suitable glycosylation, targeting and the permanent storage of protein in certain plant organs (Hood, 2002). In order to achieve gene transformation on to potato plants, an optimized shoot regeneration method is necessary. Shoot regeneration is dependent on genotype and as reported in several papers (Hoekema et al., 1989; Soto et al., 2007; Yadav and Sticklen, 1995; Yevtushenko et al., 2004). The intermodal explants of Agria and Marfona cultivars in most media introduced by past researchers were not regenerated, which was in contrast with the results achieved from their studies (Ashari and Villiers, 1998; Cearley and Bolyard, 1997; Kim et al., 2003; Soto et al., 2007). Non-desirable shoot regeneration percentage of internodal explants of Agria and Marfona cultivars was attributed to differences between the cultivars. In this study, after examination of many of these protocols, one-step medium was selected for optimization of tissue culture and shoot regeneration of Desiree, Agria and Marfona cultivars. The two last mentioned cultivars are the most commonly cultivated varieties in Iran and neighboring countries (Iranbakhsh et al., 2011; Golizadeh and Razmjou, 2010). After optimizing the shoot regeneration system, the Desiree cultivar with the average transformation rate of 30.44 was selected as the best genotype in the shortest period of time (6-7 weeks) for molecular farming and one of the best shoot regeneration media was optimized for gene transformation of Agria and Marfona cultivars, which may be useful for further studies in this field. The use of ZR as a cytokinin in the culture media reduced duration of callus phase. According to results of Beaujean et al. (1998) internodal explants were also chosen as the best explants for shoot regeneration and gene transformation, because the internodal explants had less susceptibility to injury during transformation. Moreover, variation in the different internodes on plants had less importance than the one in the leaves. In addition to human proinsulin gene transformation to Desiree cultivar, gene transformation was done to the internodal explants of Agria and Marfona cultivars due to their efficient shoot regeneration in optimized media. To our knowledge, there has been no prior optimized one-step protocol for shoot regeneration and transformation of internodal explants of Agria and Marfona in such a relatively short period of time with high shoot regeneration rate and with reduced callus phase. This protocol is applicable for petiole and leaf potato explants, without any changes and with a little increase in ZR amount, respectively. However, the frequency of shoot regeneration of Desiree, Agria and Marfona internodal explants was observed (90-100%) in 4, 9 and 9 weeks in that order. Possibly, one reason for the high percentage of shoot regeneration rate of internodal explants of Agria and Marfona in the one-step medium was its carbon source, glucose. Changing the carbon source from sucrose to glucose in the one-step medium increased the percentage of shoot regeneration rate significantly. Among carbohydrates, glucose could cause dense and green calluses that had better ability for shoot regeneration especially in Marfona and Agria cultivars. Adding mannitol along with glucose in the culture medium resulted in inducing cell growth and increasing the rate of shoot regeneration. These results for shoot regeneration of Agria and Marfona cultivars are in accordance with results of Yevtushenko et al. (2004) on shoot regeneration of Desiree cultivar. After inoculation of the explants with *Agrobacterium*, co-cultivation was done in darkness for 72 hours. Since the minimum time for Vir proteins activity and T-DNA transference to the plant cell genome, is 16 hours and a time less than 48 hours can reduce transformation efficiency and increase the co-cultivation time to more than 72 hours, can cause bacterial contamination of the explants and finally their mortality, So, 72 hours was determined as the time needed for co-cultivation. In accordance with the results of Dillen et al. (1997) and as temperatures less than 22˚C could decrease *Agrobacterium* activity and cause disorder in the process of T-DNA transference and *Agrobacterium* growth in temperatures higher than 30˚C, the co-cultivation stage was done at 22˚C and in darkness. Since light could lead the activity of the cells to the differentiation and darkness could cause the increase of cell division and in accordance with De Clercq et al. (2002), therefore co-cultivation of the internodal explants and bacteria was done in darkness. Based on the results of this research, 7.5, 12.5 and 10 mg l^{-1} of hygromycin was enough

Table 1. The mean of shoot regeneration percentage in one-step shoot regeneration media.

Variety and Media combination treatment	The mean of shoot regeneration percentage*
Agria-1	13.33
Agria-2	63.33
Agria-3	53.33
Desiree-1	53.33
Desiree-2	86.66
Desiree-3	100.00
Marfona-1	43.33
Marfona-2	30.00
Marfona-3	63.33

LSD 5%=14.39 , LSD 1%=19.71

*These numbers are the mean of shoot regeneration perce

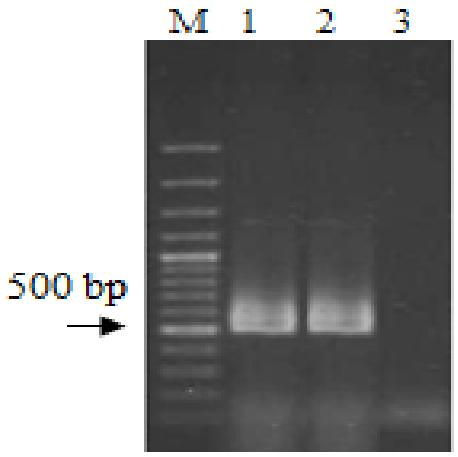

Fig 1. PCR products of Human Proinsulin gene confirm the existence of mentioned gene in Agrobacterium M: 100 bp molecular weight marker, 1: colony PCR product, 2: positive control (plasmid containing Proinsulin gene) and 3: negative control, the arrow shows a single 500 bp amplification product of transgene.

to select the transgenic internodal explants of Desiree, Agria and Marfona cultivars in that order. An extreme increase of hygromycin concentration could result in the elimination of transgenic plants. It is necessary to notice that the necessary concentration of hygromycin relied on the type of various potato cultivars. Hygromycin was able to hinder protein production in bacterial, fungal and eukaryotic cells by creating disorder in the translation process (Blochlinger and Diggelmann, 1984). Tolerance to hygromycin in transgenic plant cells is dependent on hygromycin resistance gene copy number and its location in nuclear genome. However, due to the death of non-transgenic plant cells resulting from hygromycin effect, phenol compounds and other inner vacuole constituents could negatively influence other cells such as transgenic plant cells. These cells died not only because of the selective agent, but also from the release of undesirable and poisonous components from other dead cells (Chawla, 2002). So far, various time treatments have been reported for inoculation of explants with *Agrobacterium*. In this study, using time and OD treatments showed that by increasing inoculation time and bacterium OD, it became difficult to control *Agrobacterium.* For this reason, internodal explants of Agria and Marfona cultivars were inoculated in induction media for 10 minutes and OD_{600}: 0.6-0.8 and the internodal explants of Desiree cultivar, were inoculated in an induction medium for 45 minutes and OD_{600}: 0.6-0.8. This finding was similar to results of prior research (Arif et al., 2009; Millam, 2006; Trujillo et al., 2001).

Materials and methods

Plant materials, Agrobacterium strain and used construct

In order to investigate adventitious organogenesis, the following three different potato cultivars were used: Desiree, Agria and Marfona. To produce sterile plantlets, seed tubers of Agria and Marfona were received from Seed and Plant Improvement, Karaj as well as seed tubers of Desiree from the Potato Research Center, Khorasan Razavi. Tubers cultured in the greenhouse of the agriculture faculty of Tarbiat Modares University, were first rinsed and then surface sterilized (5 minutes in 1% sodium hypochlorite and 3 subsequent washes in sterile water). Then they were put in to sterile pots filled with sterile soil, peat moss, sand and a light soil compound in a proportion of 1:1:1 for 16 hours of light, 8 hours of darkness and at a temperature of 25±1˚C. After 3-4 weeks, auxiliary buds of these plants were used to produce sterile plantlets after surface disinfection processes (70% ethanol for 30 seconds followed by 8-10 minutes in 1% sodium hypochlorite and 3 subsequent washes in sterile water). Then disinfected auxiliary buds of the greenhouse potato plants were transferred to culture media prepared in laboratory tubes for propagation. The propagation medium was a mixture of MS salts and vitamins as well as 30 gl^{-1} of sucrose and 8 gl^{-1} agar in laboratory tubes. The pH of the culture medium was adjusted to 5.8. These auxiliary buds were incubated at a temperature of 22±1˚C for 4 to 6 weeks under 16/8 hrs (day/night) photoperiod provided by cool-white florescent tubes (50 $\mu molm^{-2}s^{-1)}$. After 3 to 4 weeks, internodal segments of sterile plantlets were used as explants (Millam, 2006). The components of genotype-independent one-step medium for potato shoot regeneration were: MS salts, B_5 vitamins including nicotinic acid 1 mg l^{-1}, pyridoxine HCl 1 mg l^{-1}, thiamine HCl 10 mg l^{-1}, myo-inositol 100 mg l^{-1}, adenine sulphate 40 mg l^{-1}, Glucose 20 g l^{-1}, mannitol 20 g l^{-1} and agar 8 g l^{-1}. The pH of this medium was adjusted to 5.7. In order to determine the best concentration of ZR for shoot regeneration in this medium, the following procedure was applied: 0.05 mg l^{-1} of filter sterile GA_3 was added when the medium was autoclaved and its temperature reached to 45˚C. NAA with the amount of 0.02 mg l^{-1} was added before being autoclaved. ZR was added after being autoclaved at three levels of 2.5, 3 and 4 mg l^{-1} (Table 2). Co-culture and shoot regeneration media were used to grow *Agrobacterium* and internodal cells of potato plants simultaneously and shoot regeneration of internodal explants respectively. The components of the one-step co-culture, shoot regeneration and root induction media for internodal explants of Desiree, Agria and Marfona cultivars, are presented in Tables (3), (4) and (5), respectively. In this study, the LBA4404 strain of *Agrobacterium tumefaciens* was used for gene transformation. This strain included the expression vector of pCAMBIA1304 harboring the human proinsulin gene. This expression vector has a resistance gene against kanamycin (NPT II). It was used for expression in the bacterium and the hygromycin resistance gene was used to create hygromycin resistance in the plant. CaMV35S promoter, NOS

Table 2. The combination of plant growth regulators in one-step used for shoot regeneration of potato

Medium	Plant growth regulators		
	ZR (mgl^{-1})	NAA (mgl^{-1})	GA_3 (mgl^{-1})
M_1	2.5	0.02	0.05
M_2	3	0.02	0.05
M_3	4	0.02	0.05

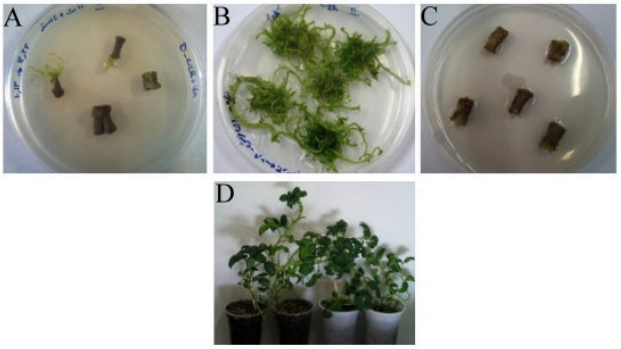

Fig 2. (A) Possibly transgenic potato internodal explants that regenerated after 4 weeks, (B) Regeneration of non-transgenic potato internodal explants (Desiree cultivar) in a culture medium without hygromycin antibiotic after 4 weeks, (C) non-regeneration of non-transgenic potato internodal explants (Desiree cultivar) in a culture medium containing 7.5 mgl-1 of hygromycin antibiotic after 4 weeks, (D) Transferring transgenic regenerated plants to the soil

transcription terminator sequence, the genes of GUS and GFP (encoding beta- glucuronidase and green florescent protein respectively) was used in front of the 35S promoter and the cutting sequences for restriction enzymes of *Bst* EII and *Nco* I that were used to replace the mentioned gene with the reporter genes. The DNA sequence of Immunoglobulin G binding protein taken from *Staphylococcus aureus* was infused to the human proinsulin gene and cloned in place of reporter genes. The fusion protein that bonded with Immunoglobulin G is called fusion protein A. This protein can cause high expression of the recombinant proinsulin gene and high stability of recombinant proinsulin protein created in the *Escherichia coli*. It is trapped chromatographically in purification methods and results in easier purification of the human proinsulin bound to it. The sequence related to fusion protein A was placed at 5′end and the sequence related to human proinsulin was placed at the 3′ end of the template sequence (Mohebodini et al., 2009) (Fig-7).

Determination of the lethal dose of hygromycin for selection

One-step regeneration media (containing 0, 5, 7.5, 10, 12.5, 15 and 20 mg l^{-1} of hygromycin) were used for determination of the lethal dose of hygromycin for selection. The non-scratched internodal explants of the 3 to 4-week-old sterile plantlets of Desiree, Agria and Marfona cultivars were put in the culture media. These internodal explants were incubated at the temperature of 22±1˚C for 4 to 6 weeks under 16/8 hrs (day/night). The photoperiod was provided by cool-white florescent tubes (50 $\mu molm^{-2}s^{-1}$).

Gene transformation procedure using Agrobacterium tumefaciens

About 4 to 6 millimeters of each 3 to 4-week-old internodal explants of Desiree, Agria and Marfona plantlets, (diameters not less than 2 mm) were used for gene transformation. Suspension culture of *Agrobacterium* harboring the target gene ($OD_{600\ nm}$= 0.6-0.8) was prepared by incubation at 28 ˚C on an orbital shaker at a rotating speed of 200 rpm. After centrifuging 1300 ×g for 5 minutes, the bacterium pellet was suspended in 20 ml of liquid induction culture medium having 20 mg l^{-1} of acetosyringone. Suspension culture of *Agrobacterium* again was put in a 28˚C shaker incubator at a rotating speed of 200 rpm for 2 hours. The internodal explants of mentioned cultivars were put in this bacterial suspension (on an orbital shaker at a rotating speed of 50 rpm) for 45 minutes. The internodal explants were dried on sterile filter papers and kept in darkness for 3 days in a co-culture medium including: MS salts, B_5 vitamins, 40 mgl^{-1} Adenine sulphate, 20 gl^{-1} glucose, 20 gl^{-1} mannitol, 0.05 mgl^{-1} GA_3, 0.02 mgl^{-1} NAA, (3, 2.5 and 4 mgl^{-1} ZR for Desiree, Agria and Marfona cultivars in that order) and 8 gl^{-1} agar. At the next stage, the infected internodal explants were transferred to the selective culture medium with 500 mg l^{-1} cefotaxime and 7.5, 12.5 and 10 mg l^{-1} hygromycin for Desiree, Agria and Marfona cultivars respectively. They were incubated at a temperature of 22±1˚C for 4 to 6 weeks under a 16/8 hrs (day/night) photoperiod provided by cool-white florescent tubes (50 $\mu molm^{-2}s^{-1}$). Small regenerated shoots were cut and transferred into bigger glass bottles containing MS based extending and root-generating culture medium including 20 g l^{-1} sucrose as well as 500 mg l^{-1} cefotaxime and 7.5, 12.5 and 10 mg l^{-1} hygromycin (for Desiree, Agria and Marfona cultivars respectively). These glass bottles were incubated at a temperature of 22±1 ˚C for 16/8 hours (day/night) for 3 weeks. Finally, the potato plantlets were transferred into pots containing vermicolite (50%), peat and perlite (25% each) in preparation for transfer to the soil.

Genomic DNA extraction and DNA analysis of the transgenic plants

In order to extract genomic DNA, young green leaves from the first internodes of 6-week-old potato plants were used and genomic DNA was extracted from them using modified CTAB (Porebski et al., 1997). The existence of the target gene was confirmed in the transgenic plants by carrying out PCR, using specific primers. About 50-100 ng of a template DNA, forward and reverse primers, $MgCl_2$, PCR buffer, dNTPs and *Taq* DNA polymerase at a concentration of 0.4 µmol, 1.4 mM, 1X, 0.2 mM and 1.5 units were used respectively to carry out PCR. PCR was performed in the following conditions: 1 cycle at hot start, 94˚C/300s; 35 cycles as follows:
Denaturation, 94˚C/40s; annealing 57˚C/40s; extension 72˚C/35s and finally the reaction mixtures were placed for 200s at 72˚C. The specific primers used for amplification of the DNA sequence of protein A and human proinsulin were:
5′-CATGCCATGGAAGCGGGATTCAACCAATTTAATA-AGG -3′ and 5′-CCGGTCACCTCATTAGTTGCAGTAGT-TTTCCAG-3′.

Table 3. The components used in co-culture, shoot regeneration, root induction and shoot elongation medium for transformation of Desiree cultivar.

Components	Concentration in medium		
	co-culture	shoot regeneration	root induction and shoot elongation
MS salts	1X	1X	1 X
B_5 vitamins	1X	1X	-
MS vitamins	-	-	1 X
Adenine Sulphate	40 mgl^{-1}	40 mgl^{-1}	-
Glucose	20 gl^{-1}	20 gl^{-1}	-
Mannitol	20 gl^{-1}	20 gl^{-1}	-
Sucrose	-	-	30 gl^{-1}
GA_3	0.05 mgl^{-1}	0.05 mgl^{-1}	-
NAA	0.02 mgl^{-1}	0.02 mgl^{-1}	-
ZR	3 mgl^{-1}	3 mgl^{-1}	-
Agar	8 gl^{-1}	8 gl^{-1}	8 gl^{-1}
Cefotaxime	-	500 mgl^{-1}	500 mgl^{-1}
Hygromycin B	-	7.5 mgl^{-1}	7.5 mgl^{-1}

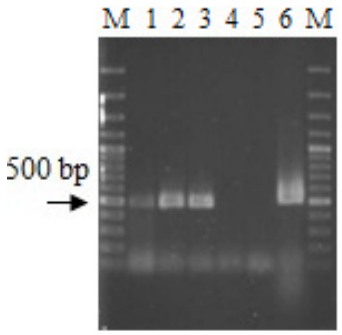

Fig 3. PCR products of Human Proinsulin gene confirm the existence of mentioned gene in transgenic plants. M: 100 bp molecular weight marker, 1, 2 and 3: PCR product of transgenic lines, 4: blank control (with ddH2O as a template), 5: negative control (with DNA of non-transgenic plant as a template) and 6: positive control (with the plasmid harboring target gene as a template), the arrow shows a single 500 bp amplification product of transgene.

RT-PCR analysis

In order to extract RNA, RNX^{TM} (-plus) (Sina-Gene Co.) was used. To ensure against DNA contamination, DNase I Omission Kit was used for the RNA extracted samples. Therefore, in each extraction, RNA samples were treated by DNase I before cDNA formation. This procedure was performed in the following conditions: Incubation 30 minutes at 37˚C, using 1 µl of RQ_1DNase stop solution in order to end the reaction and finally incubation for 10 minutes at 60˚C, to stop activity of DNase. cDNA synthesis was done using RT-PCR Kit (Fermentas) according to the manufacturer's instructions and used for cDNA sequence amplification using respective primers as described above.

Protein analysis of the transgenic plants
SDS-PAGE assays

200 mg of young potato leaves were used to extract total soluble protein (TSP). The potato leaves were ground in liquid nitrogen to form a fine powder. Soluble proteins were extracted using 1000 µl of extraction buffer (50 mM Tris-HCl, 2 mM Ethylene Diamine Tetra Acetic Acid (EDTA) and 0.04% (v/v) 2- Mercapthoethanol). Cell debris was removed by two rounds of centrifugation (in 24000 g, 21 min at 4 ˚C) and the supernatant was used for sodium-dodecyl sulphate polyacrylamid gel electrophoresis. SDS-PAGE of proteins was done using 14% polyacrylamid gel followed by staining with Coomassie blue (Laemmli, 1970).

Enzyme linked immunosorbent assay (ELISA)

The double antibody sandwich ELISA (DAS ELISA) procedure was performed according to the general method described by Clark and Adams (Clark and Adams, 1977). ELISA plates (JET BIOFIL) were coated with 100 µl of the recommended dilution of IgG in a carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 5 mM NaN_3, pH: 9.6) and incubated overnight at 4˚C. Plates were washed four times at 5 min intervals with a washing buffer (PBS: 2.7 mM KCl, 3 mM NaN_3, 8 mM Na_2HPO_4, 1 mM KH_2PO_4 and 0.13 M NaCl in addition 0.05% Tween 20) and 100 µl (20 ng) of plant protein extract was added to each well and incubated overnight at 4˚C. (Protein concentration was determined by the Bradford Protein Assay Reagent Kit (Bio-Rad) with BSA as a standard). After washing the plates, wells were incubated with 1: 200 dilution of rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) in PBS-T (1 h at 37°C). Wells were washed three times with PBS-T and incubated (1 h at 37°C) with a 1:1000 dilution in PBS-T of anti-rabbit IgG alkaline phosphatase conjugate (Sigma A3687). Plates were developed with 100 µl *p*-nitrophenyl phosphate liquid substrate (Sigma N7653) per well. Reactions were stopped with 1 N HCl, and the absorbance was determined at 405 nm using ELISA- reader (BioTek Elx 800). A sample was considered positive (transgenic) if absorbance at 405 nm was greater than or equal to three times the average of negative (non-transgenic) samples. To quantify protein expression 0.5, 1.5, 2.5, 5, 10 and 20 ng μl^{-1} of purified insulin was added in 6 wells as samples. The absorbance of these positive samples at 405 nm was used for drawing calibration curve.

Dot-blot immunoassay of human proinsulin

Dot-blot was only used to show presence of the human proinsulin protein in transgenic lines. According to the methods described earlier (Banttari and Goodwin, 1985), each leaf protein extract was dotted on the nitrocellulose membrane (0.45 µm pore size, GIBCO, Grand Island, NY), and then the membrane was blocked for 1 h at room temperature (25˚C) in 2% BSA in PBS-T. The nitrocellulose membrane was washed three times in PBS-T and incubated in a 1: 200 dilution of primary antibody in PBS-T for 1 h, washed three times and incubated with 1: 4000 dilutions of anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.) as a secondary antibody for 1 h. Color development solution was 0.05% 3, 3'-diaminobenzidine tetrahydrochloride or DAB (Santa Cruz Biotechnology, Inc.) and 0.01% hydrogen peroxide in 50 mM Tris (pH 7.5). All buffers in this method contained Sodium Azide as a preservative. Development of a brown color in each dotted area was interpreted as a transgenic line.

Statistical analysis

Experiments were established in a factorial experiment based on completely randomized design. Three replications per

Table 4. The components used in co-culture, shoot regeneration, root induction and shoot elongation medium for transformation of Agria cultivar

Components	Concentration in medium		
co-culture	shoot regeneration	root induction and shoot elongation	
MS salts	1X	1X	1 X
B_5 vitamins	1X	1X	-
MS vitamins	-	-	1 X
Adenine Sulphate	40 mgl^{-1}	40 mgl^{-1}	-
Glucose	20 gl^{-1}	20 gl^{-1}	-
Mannitol	20 gl^{-1}	20 gl^{-1}	-
Sucrose	-	-	30 gl^{-1}
GA_3	0.05 mgl^{-1}	0.05 mgl^{-1}	-
NAA	0.02 mgl^{-1}	0.02 mgl^{-1}	-
ZR	2.5 mgl^{-1}	2.5 mgl^{-1}	-
Agar	8 gl^{-1}	8 gl^{-1}	8 gl^{-1}
Cefotaxime	-	500 mgl^{-1}	500 mgl^{-1}
Hygromycin B	-	12.5 mgl^{-1}	12.5 mgl^{-1}

Table 5. The components used in co-culture, shoot regeneration, root induction and shoot elongation medium for transformation of Marfona cultivar.

Components	Concentration in medium		
co-culture	shoot regeneration	root induction and shoot elongation	
MS salts	1X	1X	1 X
B_5 vitamins	1X	1X	-
MS vitamins	-	-	1 X
Adenine Sulphate	40 mgl^{-1}	40 mgl^{-1}	-
Glucose	20 gl^{-1}	20 gl^{-1}	-
Mannitol	20 gl^{-1}	20 gl^{-1}	-
Sucrose	-	-	30 gl^{-1}
GA_3	0.05 mgl^{-1}	0.05 mgl^{-1}	-
NAA	0.02 mgl^{-1}	0.02 mgl^{-1}	-
ZR	4 mgl^{-1}	4 mgl^{-1}	-
Agar	8 gl^{-1}	8 gl^{-1}	8 gl^{-1}
Cefotaxime	-	500 mgl^{-1}	500 mgl^{-1}
Hygromycin B	-	10 mgl^{-1}	10 mgl^{-1}

treatment with 10 explants for each replication were used. The experiment was repeated twice. Data (shoot regeneration percentage) were reported as the mean of two experiments. Percentages of shoot regeneration were calculated for internodal explants that had been cultured for 4 weeks. Analysis of variance was used to test the significance of the effects of genotype and plant growth regulator concentrations for shoot regeneration. The means of treatment combinations were compared using LSD test. Data was subjected to analysis of variance and means compared using the SPSS program version 14.0 (2005). Normality tests for data were assessed using the Anderson- Darling normality test with Minitab 14 (2005).

Conclusion

Producing a single 500 bp amplification product using PCR technique showed that potato plants were transgenic. RT-

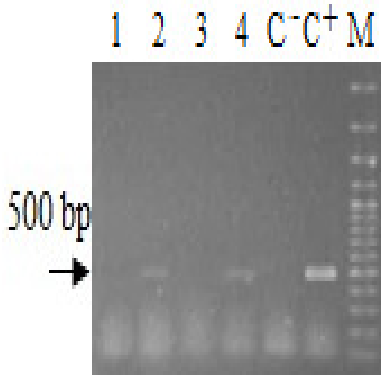

Fig 4. RT-PCR products of Human Proinsulin gene confirm the existence of mentioned gene in RNA level in the transgenic plants. M: 100 bp molecular weight marker, 2 and 4: RT-PCR product of transgenic plants (that their DNA analysis was positive). 1 and 3: the negative controls (that in which the extracted RNA of transgenic plants of lanes no. 2 and 4 were applied as a template). C-: blank (negative control). C+: positive control, the arrow shows a single 500 bp RT-PCR product of transgene.

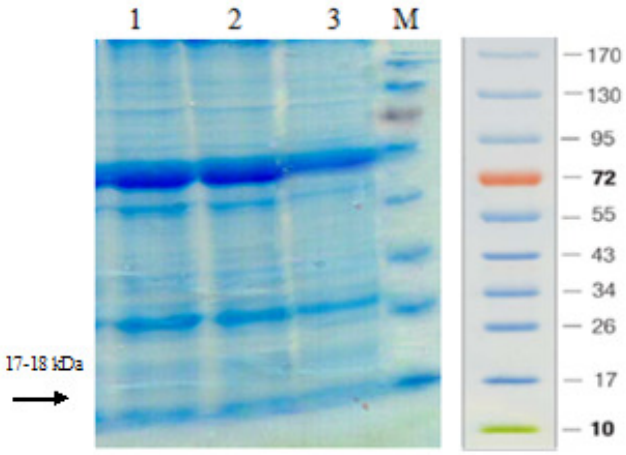

Fig 5. The protein analysis of the transgenic plants using SDS-PAGE method M: Molecular weight marker (Fermentase, SM 0671), 1 and 2: transgenic plants (that RNA and DNA analysis was positive) and 3: the control plant that is non-transgenic, arrow shows the band of recombinant protein at 17-18 kDa.

PCR, SDS-PAGE, ELISA and dot-blot analysises confirmed the human proinsulin gene expression at RNA and protein levels. As mentioned, increasing demand for insulin is predicted for the near future. Therefore, insulin production methods that improve efficiency, safely and at low-cost are necessary and potato is one of the best available bioreactors. Recombinant proinsulin expression in transgenic potato plant cells (0.022% TSP), strongly depends on gene copy number and its location in nuclear genome. CaMV35S is the most commonly used constitutive promoter for high levels of gene expression in dicot plants and our purpose was confirmation of proinsulin expression in different variety of potato

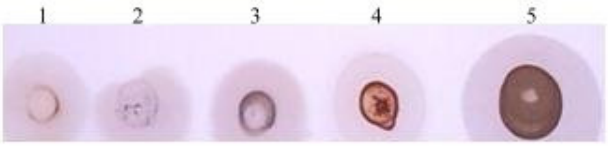

Fig 6. Dot-blot immunoassay of human proinsulin Proteins extracted from putative transgenic plants are spotted onto membrane and hybridized with antibody probe.1: positive control, 2: negative control, 4 and 5: transgenic lines. 3: probably non-transgenic line.

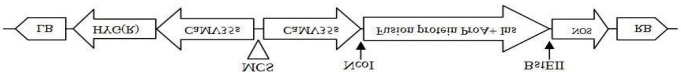

Fig 7. Genes located within the T-DNA flanked by the right and left border (RB and LB) are inserted into the plant genome and include: CaMV35S, CaMV35S promoter; ProA+ ins, Protein A-Proinsulin fusion; HYG(R), hygromycin phosphotransferase; NOS, nopalin synthase terminator; MCS, Multiple cloning site.

especially Agria and Marfona cultivars which are the most commonly cultivated varieties in Iran. To increase proinsulin gene expression, introducing modification in this construct and producing other constructs with tuber-specific promoters or with enhancer sequences has probably made it possible to achieve higher expression levels.

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