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Original scientific paper

TRANSFER OF HUMAN PROINSULIN GENE INTO CUCUMBER (*Cucumis sativus* L.) VIA *AGROBACTERIUM* METHOD

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Nowadays, approximately 5.8% in adult population around the world are suffering by diabetes. It can be caused by an increase in risk factors such as being overweight. Also it has been estimated that the number of patients will be doubled in near future and the demands for insulin hormone will be growing up by 3 to 4 % annually. Therefore, it's necessary to develop new methods for hormone production with high rate of capacity in future. By advanced technology of transgenic DNA, the transgenic plants are introduced as an attractive system for expression and production of many kinds of pharmaceutical proteins. In this study, we investigated transfer of Human Proinsulin Gene into the Cucumber (*Cucumissativus* L.). Transgenic cucumber could be a great prospect for future source of eatable insulin pharmaceutical drugs to be taken by patients.*Agrobacterium tumefaciens*strain *LBA4404* carrying proinsulin genes with CaMV 35S promoter was used for the transformation purpose. The transgenic plants were analyzed by PCR, RT-PCR, SDS-PAGE, Dot blot and Electrochemiluminescence techniques. Production of proinsulin in cucumber could be a great prospect in molecular farming of human proinsulin.

Keywords: Gene expression analysis, Human proinsulin transformation, Recombinant pharmaceutical protein, transgenic cucumber

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INTRODUCTION

Diabetes mellitus type 1 (T1DM, IDDM, or juvenile diabetes) is a form of diabetes mellitus that results from autoimmune destruction of insulin-producing beta cells of the pancreas The subsequent lack of insulin leads to increased blood and urine glucose that requires insulin injection to compensate the problem. According to 2011's report of *World Health Organization* Type 1 diabetes causes an estimated 11–22 million cases worldwide and also the incidence of the disease has been increasing by about 3% per year. As diabetes is rapidly becoming one of the world's most common diseases, its financial cost is also increasing(AANSTOOT ANDERSON *et al*., 2007). However, a lot of researches have been carried out for finding an effective treatment for this disease, and scientists in many countries are still striving to reach a suitable solution. The insulin injection has been the only effective way to cure diabetes type 1, but the difficulty of this injection has been a major source of patients' dissatisfaction. By increasing the number of diabetic patients, the demands for insulin hormone are rising by 3 to 4 % annually. Owing to this fact, it's necessary to provide new methods for hormone consumption and to develop economical and improved approaches with high production capacity in future.

Based on recent researches, are introduced as an attractive bioreactor for producing recombinant pharmaceutical proteins compared with other production systems. Nowadays, many biopharmaceutical proteins and peptides have been produced in transgenic plants (ERICKSON *et al*. 2002). In more recent studies, the human insulin was expressed in transgenic *Arabidopsis* seeds (NYKIFORUK *et al*., 2006; MOHEBODINI *et al.,* 2014). The Glucagon-like peptide-1 (GLP-1) was transformed into cucumbers and biological activity test results showed that the serum glucose level of diabetic rats was significantly decreased after oral administration of transgenic cucumber fruitage (ZHAO *et al*,. 2009).

The cucumber is one kind of preferred food for diabetic patients due to its low sugar content, good taste and a wide range of cultivation (ZHAO *et al.,* 2009).It was also shown in a research that cucumber (*Cucumissativus* L.)significantly decreased the area under glucose tolerance curve and the hyper glycaemicpeak in healthy rabbits subjected to a weekly oral glucose tolerance test (BANERJEE and BASU 1991).In this paper, transfer and expression of Human Proinsulin Gene in the Cucumber (*Cucumissativus* L.) was studied.

MATERIALS AND METHODS

Plant materials

Seeds of Isfahan's cucumber cultivar were provided from The Iran Seed and Plant Improvement Institute. Due to the fact that there was no report of Iranian's cucumber cultivar tissue culture, in this study, at first tissue culture and direct regeneration of Isfahan's cucumber cultivar was optimized via testing of many different hormones' treatments. Then, the best medium and hormones were selected and used in regeneration medium (3mg/L BAP and 0.1 mg/L NAA in MS medium).

Bacterial strains and plasmid construct

Human Proinsulin gene cloning in plant expression binary vector pCAMBIA1304 were done as in (MOHEBODINI *et al.* 2014). DNA of the binary vector (pCAMINS) was transferred into the *A. tumefaciens* strain LBA4404 by the freeze-thaw method. The constructed vector (pCAMINS) was confirmed by different methods like colony PCR, PCR, digestion and sequencing.

The sensitivity of explants to Hygromycin

In order to find the inhibitory concentration of Hygromycin, different levels of Hygromycin were studied. The sensitivity of cotyledon explants to Hygromycin was determined by culturing the explants in regeneration medium (MURASHIGE and SKOOG (MS) medium and 3mg/L 6-Benzylaminopurin (BAP) and 0.1 mg/L Naphthaleneacetic Acid (NAA)) along with 5 to 25 mg/L Hygromycin. Moreover, a positive control without Hygromycin was maintained.

Production of transgenic cucumber plants

Transgenic cucumber plants were produced by *Agrobacterium tumefaciens*-mediated transformation. *In vitro* raised seedlings on MS medium were used as an explants source. Cotyledonary explants (proximal- 0.5 cm) from 6 days old cucumber seedlings were inoculated with *Agrobacterium tumefaciens* containing pCAMINS (OD_{600} nm =1) for 10 minutes. Then, inoculated explants were blotted with sterile Whatman No. 1 filter paper and then were placed in co-cultivation medium (MS medium) and were kept at a temperature of 28 ° C in dark for 48 hours. After 2 days, explants were transferred to the selective shoot regeneration medium based on MS medium containing, 3% sucrose, 0.8% agar, 3mg/L BAP and 0.1 mg/L NAA along with 200 mg/L Cefotaxime and 15 mg/L Hygromycin B for selective transformed cells. Furthermore, the inoculated explants were kept at 24±2°C under 16-h photoperiod. After one month, when the putative transformed shoots developed to 1.5 cm in length, the regenerated Hygromycin resistant shoots were separated from explants and cultured into a MS medium containing 200mg/L Cefotaxime with Hygromycin antibiotic for rooting of transgenic cucumber plants. When the regenerated plants matured, they were transferred and maintained in a greenhouse.

Transgenic cucumber analysis

PCR analysis

Genomic DNA from transformed and untransformed (control) plants was extracted by cetyltrim ethylammonium bromide (CTAB) method (MURRAY and THOMPSON, 1980) and used as the template for PCR analysis. For amplification of pro-insulin gene, two primers were used as following Forward primer, 5'-AAGCGGGATTCAACCAATTTAATAAGG -3' Reverse primer,5'-TCATTAGTTGCAGTAGTTTTCCAG -3. The PCR program was one cycle of 94°C for 30 seconds followed by 35 cycles of 94°C for 40 seconds, 57°C for 40 seconds and 72°C for 35 seconds. Finally, the last cycle was continued in 72°C for 20 seconds. According to touchdown PCR results, the best annealing temperature was 57 °C. PCR produced a fragment with an approximate size of 458 bp.

Reverse transcription –PCR analysis

For analysis at transcriptional level by RT-PCR (Reverse Transcription PCR), first Total RNA from transformed and untransformed (control) plants was extracted by using a RNXTM (-Plus) kit (Cinna Gen Co.). After extraction, RNA's quality was checked by electrophoresis. First-strand cDNA of the antisense transcripts was synthesized using Revert AidTM H Minus M-MuLV Reverse Transcriptase enzyme and gene-specific primer. Total RNA (5 µg) was incubated with 10-15 pmol primers and 0.5 mm dNTPs at 70°C for 5 minutes and immediately chilled on ice. Then, the reaction buffer, RNase inhibitor and Reverse Transcriptase enzyme (200U/µl) were added to give a final concentration. The mixture was incubated at 42°C for 60 minutes and after that the

reaction was stopped by heating the mixture at 72°C for 10 minutes. The cDNA product was used as template for a RT-PCR reaction with gene-specific primer.

Protein analysis

Protein extraction

The transgenic cucumber leaves were powdered in liquid N_2 and the total protein was extracted by Guy method (GUY *et al*., 1992). For the extraction of total soluble protein (TSP), 200 mg of young transgenic cucumber leaves were ground into powder. The soluble protein was extracted by using 1000 µl of extraction buffer (50 mMTris-HCl, 2 mM Ethylene Diamine Tetra Acetic Acid (EDTA) and 0.04% (v/v) 2- Mercaptoethanol) and centrifugation at 12000×g for 20 minutes at 4°C. Then, the supernatant was transferred into a new tube. The supernatant was used as an extracted protein.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) assays

SDS-PAGE of proteins was performed using 10% Polyacrylamid gel followed by staining with Coomassie blue (LAEMMLI 1970). Leaves extract (5–10 µg protein as determined by Bradford assay), boiled for 5 min with β-mercaptoethanol, and were electrophoreses in a 14% (w/v) SDS–PAGE.

Dot blot analysis

Dot blot was used to show the expression and presence of human proinsulin protein in transgenic lines. For dot blot analysis, each leaf protein extract was blotted on the nitrocellulose membrane (0.45 µm pore size, GIBCO, Grand Island, NY), and then the membrane was blocked for 1 hour 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 (v/v) of rabbit polyclonal antibody as the primary antibody (Santa Cruz Biotechnology Inc) in PBS for 1 hour, washed three times and incubated with 1: 4000dilutions of Alkaline Phosphates-conjugated goat ant rabbit in PBS for 1 hour. Color development solution was 3, 3´- diaminobenzidine(DAB) in PBS buffer containing 50 µl of H_2O_2, and 50 µl of 3, 3´- Diaminobenzidine per 1 ml PBS buffer prepared just before use. All buffers in this method contained Sodium Azide as a preservative. Development of brown color in each dotted area was interpreted as a transgenic line.

Electrochemiluminescence (ECL)

For doing the Electrochemiluminescence test, the specific insulin kit of ECL was used according to the manufacturer's protocol (Meso Scale Discovery, Gaithersburg, USA). The Human Insulin Kit provides assay-specific components for the simultaneous quantitative determination of recombinant human insulin in total soluble protein.

RESULTS

Sensitivity of cotyledon explants to Hygromycin

The different concentrations (5 to 25 mg/L) of Hygromycin were employed to find out the suitable inhibitory concentration for Isfahan's cultivar regeneration. The maximum shoot regeneration was obtained at 5 mg/L Hygromycin. In the media containing 15 mg/L hygromycin all the explants became brown and died. Therefore, 15 mg/L hygromycin was used to select transformed shoots in this study.

Production of transgenic plants

Six days old cotyledons from Isfahan cucumber cultivar were incubated with *Agrobacterium tumefaciens* containing pCAMBINS plasmid for two days. Then they were transferred to the shoot regeneration medium including 200 mg/L cefotaxime and 15 mg/L Hygromycin (optimum concentration). In Isfahan cucumber cultivar, the optimum of inoculum for inoculation was determined 1 OD and the best co-cultivation period was evaluated as two days in hormone free MS medium. After 3 weeks, at the end of some proximal cotyledon explants, the small green shoot bud were started to regenerate. However, there was no regeneration on the control explants (Figure 1a).

After one month, to prevent Hygromycin inhibition effect on shoot but elongation, the regenerated shoots in the selected medium were separated and transferred to the MS medium with 200 mg/L cefotaxime. Putative transgenic shoots were developed and rooted in new culture medium (Figure1.b). In vitro rooted plantlets were acclimatized and cultivated in greenhouse in lightweight soil mixture of Coco Peat and Perlite (Figure1.c).

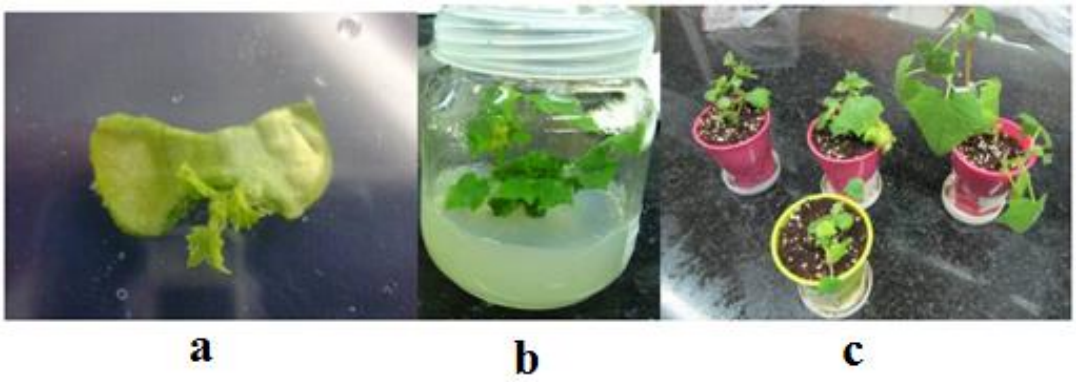

Figure.1. Different growth stages of transgenic Cucumber (*Cucumis sativus* L.) plant. a. Direct regeneration from inoculated cucumber explants. b. Rooting of transgenic plants. c. Transfer of transgenic plants to soil.

Transgenic plants analysis

The transgenic plants were analyzed in three stages: at DNA, RNA and protein level.

PCR analysis

Analysis of the plants that regenerated in the selection medium by PCR amplification confirmed the presence of proinsulin gene in the putative transformed plants. The DNA isolated from putative transformed and wild type plants and plasmid containing proinsulin gene was used as DNA templates in PCR analysis. The presence of amplified band at 500 bp in putative transformed shoots proved the presence of proinsulin gene while no amplification was detected in wild type plants (Figure 2).

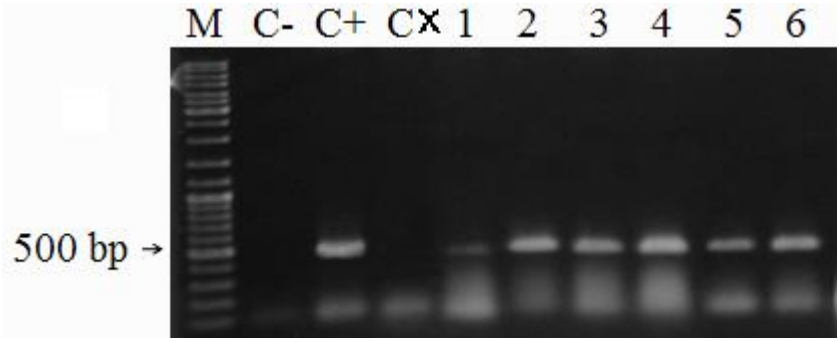

Figure 2. PCR products of transgenic cucumber plants. M: 100 bp marker, C- : PCR content without DNA, C+: Positive control (pCAMINS plasmid) C× : Wild type Plant, Lanes 1-6: specific 0.5-kb PCR band in transgenic cucumber plants.

RT–PCR analysis

The PCR product with expected size was detected following reverse transcription of the total RNAs extracted from leaf tissues of transgenic cucumber plants. The results indicated that the gene was actively transcribed (Figure 3).

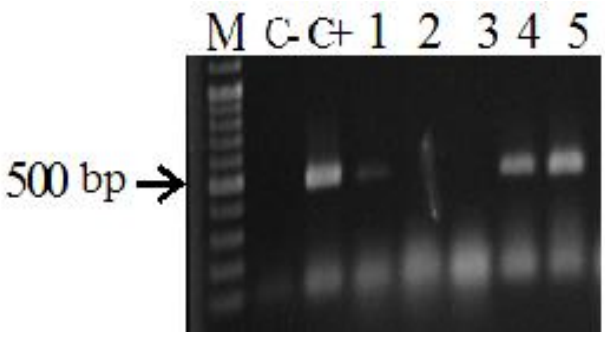

Figure 3. Evaluation of proinsulin gene expression in transgenic cucumber using RT-PCR. M: 1 kb marker, C- : Negative control with the RNA extracted from the Wild type Plant as a template, C+: Positive control (pCAMINS plasmid), Lanes 1, 4 and 5: RT-PCR product of transgenic plants (that their DNA analysis was positive), lanes 2 and 3: cucumber plants that resulted negative for RT-PCR.

SDS-PAGE analysis

Total soluble protein from wild type and transgenic leaves was loaded into a SDS-PAGE gel. The protein bands on SDS gel were compared. As a result, the predicted band of proinsulin about 17 kDa (an inconspicuous and pale band) was detected (Figure 4).The detected band could not be

used as an integrity confirmation of the proinsulin protein expression; therefore, the extracted proteins were further analyzed by Dot blot and Electrochemiluminescence methods.

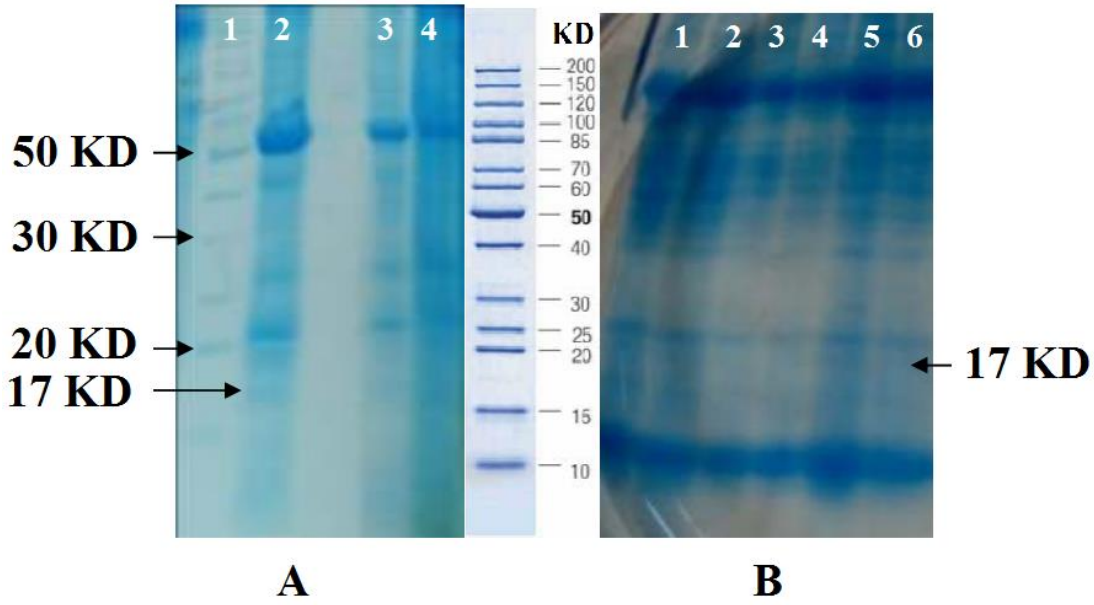

Figure 4. SDS-PAGE of protein extracted from transgenic leaves compared with wild type. A: 1: 200 kDa marker. 2: Transgenic cucumber plant protein extract, 3, 4: Wild type cucumber protein extract. B:M, 200 kDa marker, 1,2 , 5, and 6.,Transgenic cucumber plant protein extract. , 3, 4: Wild type cucumber protein extract.

Dot blot

Positive PCR plants were further tested for detection of proinsulin protein by Dot blot analysis with using rabbit anti-insulin polyclonal antibody. Insulin protein was used as positive control. The result of Dot blot and comparison of the transgenic with wild type plants, were demonstrated the expression of proinsulin protein (Figure 5).

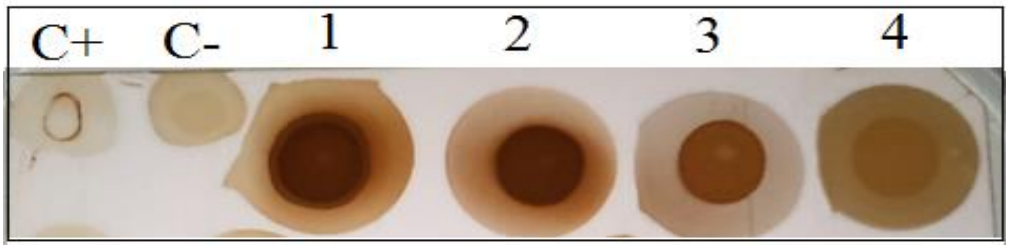

Figure 5. Dot blot analysis of proteins extracted from leaves of transgenic and wild type plants C+: positive control (pure insulin), C- negative control (protein extracted from leaves of wild type plant). 1, 2, 3, and 4: positive results of four transgenic cucumber protein

Electrochemiluminescence

Total soluble protein from leaves of transgenic and wild type plants were extracted and analyzed by Electrochemiluminescence method using specific insulin kit. The minimum amount of measuring of insulin protein in this method is 1.39 pmol/L. The test result was shown the amount of 7.18 and 6.87 pmol/L in two samples and confirmed the proinsulin protein presence and expression in transgenic cucumber.

DISCUSSION

Plants are introduced as suitable candidates for recombinant biopharmaceutical productions. They have several advantages like lower production cost, more safety and absence of toxic compounds in comparison with other foreign protein producer organisms (such as bacteria, fungi and mammalian cells) (DANIELL *et al.*, 2001; GIDDINGS 2001; SUGITA *et al.*, 2005).On the other hand, rising demand for insulin hormone and the need to find some alternative ways for intensification of insulin production make it a necessary candidate.

In this study, we investigated transformation of proinsulin gene into Iranian's cucumber cultivar as a bioreactor to produce proinsulin protein and as a way for improving production of insulin in future time. In case of gene transformation, 5-days cotyledons were incubated with *Agrobacterium tumefaciens* suspension for 10 minutes. In this duration, we have obtained suitable transformation rate like other researches (SONIYA and DAS 2002; SELVARAJ *et al.* 2007; VASUDEVAN *et al.*, 2007; ZHAO *et al.*, 2009). In the regeneration phase, the best hormone for direct shoot regeneration and induced multiple shoots were obtained at high level of BAP (3mg/L) while in other reports low level concentrations of BAP (about 0.5 to 1 mg/l) had been used (GANAPATHI and PERL-TREVES 2000; SAPOUNTZAKIS and TSAFTARIS 2000). Therefore, it isconfirmed the importance and dependence of genotype role in success of cucumber regeneration as reported by Wehner and Kim (KIM *et al.*, 1988).

Regeneration procedure is the most important requirement for reliable plant transformation, and for the first time, we could obtain an efficient and stable way of regeneration for Isfahan's cucumber. The transformation efficiency is defined as percentage of producing regenerated shoots or transgenic plants (YIN *et al.*, 2005). The percentage of cucumber transformation efficiencies are relatively low in reports (about 1.4 to 10%) for regenerated shoots (NISHIBAYASHI *et al.*, 1996; TRULSON *et al.*, 1986; CHEE 1990; VASUDEVAN *et al.*, 2007).

In this investigation, the 28% of regenerated shoots were rooted on the rooting medium. All of the rooted plants were transplanted to pots containing mixture of *Cocopitand Perlite* and the 38% of rooted transgenic plants were survived and acclimatized in greenhouse.

The expression of proinsulin gene was analyzed on DNA, RNA and protein level. Proinsulin gene transformation confirmed by PCR and it has shown that at least one copy of gene was transferred into the cucumber genome. In the next level, we investigated proinsulin expression in RNA level by RT-PCR and the result showed the new transformed gene expression at mRNA level. However, in some positive PCR plants, the mRNA wasn't found in RT-PCR analysis that could be due to reasons such as low expression, gene silencing, positional effect, DNA methylation, chromatin components and location of new integrated gene (MEYER 2000, GELVIN 2003).

We analyzed protein level for all transgenic plants with PAGE, Dot blot, and Electrochemiluminescence methods: SDS-Based on SDS-PAGE, the difference between proteinsin non-transgenic cucumber plant and transgenic cucumber plants have been observed as a faint band

(17kDa) at the expected district. This band could be specific band of Fusion A protein with recombinant proinsulin. The faintness of observed band probably was because of low levels of recombinant protein compared with natural proteins of plant.

Dot blot is an exact method for analysis of protein levels (BANTTARI and GOODWIN 1985). In our study, recombinant protein expression and the presence of proinsulin in transgenic cucumber plants were positively confirmed by this method. Dot blot was only used to demonstrate the presence of human proinsulin recombinant protein in transgenic samples. Although ELISA methodizes common way for immunologic reaction using an antibody against the antigen, but ELISA and Chemiluminescence methods are not perfect and have some disadvantages of being unable to measure very low concentrations of the proteins. In order to solve Immunoassay problems, Electrochemiluminescence method has been developed in the past few years (MATHEW *et al.,* 2005).

Golla and Seethala compared the rate of identify edratinsulin in two ELISA and ECL methods(GOLLA and SEETHALA 2004). The related results were proved that the insulin assay with ECL is much more efficient than other current measuring methods. Also, they stated that the ECL was a very accurate with high sensitivity, low cost and reliable to determine the amount of insulin. Also, another test showed that ECL is four times more sensitive and faster than the ELISA assay(GUGLIELMO-VIRET *et al.* 2005).In this study, the obtained experimental results by ECL method were confirmed the expression and presence of recombinant proinsulin protein in transgenic cucumber plants with very high accuracy.

The amount of 7.18 pmol/L of recombinant proinsulin protein was reported by ECL. Based on the extracted amount of total protein from 0.2 gleaf tissue and our calculations, there could be at least 650 microgram sproinsulinin1 kg offers leaf tissue of transgenic cucumber plants. In another experiment, the amount of total protein extracted from leaf tissue was measured by Bradford method (data not shown) and compared with amount of the obtained recombinant proinsulin protein by ECL method. As a result, the percentage of recombinant protein in total protein of transgenic cucumber leaf tissue was about 0.01%.This low amount of recombinant protein production could be due to many reasons, such as recombinant protein degradation by plant protease or transfer red gene being satin the region of genome that has impossible or very low expression (PRASAD *et al.* 2004, DORAN 2006).In various studies performed in transgenic plants,productconcentrationof0.01% to 0.1% of total protein and even less has been reported (DANIELL *et al*. 2001). The amount of proinsulin protein in present study is about the same range as in other investigations, and this amount of expression is evaluated to be acceptable range of nuclear gene transformation (MOHEBODINI *et al.* 2014).

In conclusion, we can say the present protocol and it could be applied to improve cucumber transformation via *Agrobacterium tumefaciens*. This may provide a new strategy to diabetes treatment without pain compared with insulin injections. More detailed studies are ongoing in our laboratory to assess the effects of transgenic cucumber on diabetes mellitus, increasing the recombinant protein expression, and its bio-safety factors.

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TRANSFER HUMANOG PROINSULIN GENA U KRASTAVAC (*Cucumis sativus* L.) POMOĆU *AGROBACTERIUM* METODA

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Izvod

Danas, približno 5.8% odrasle populacije u svetu boluje od dijabetisa. To može biti uzrokovano povečanim faktorima rizika kao što je gojaznost. Takođe se procenjuje da broj pacijenata će se udvostručiti u bliskoj budučnosti i da će zahtevi za insulinom da sepovečavaju do 3 - 4% godišnje. Zato, je neophodno da se razvije novi metod za proizvodnju hormona sa visokom stopom kapaciteta u budučnosti. Pomoću napredne tehnologije transgene DNK transgene biljke su alternativni sistemi za ekspresiju i proizvodnju mnogih farmaceutskih proteina. U ovom radu smo ispitivali prenos humanog proinsulin gena u krastavac (*Cucumissativus* L.). Transgeni krastavac bi mogao biti izvor jestivog insulina. *Agrobacterium tumefaciens* soj *LBA4404*, vektor proinsulin gena sa CaMV 35S promotorom je korišćena za transformaciju. Transgene biljke su analizirane PCR, RT-PCR, SDS-PAGE, Dot blot i Electrochemiluminescence tehnikama. Proizvodnja proinsulina može biti od značaja u molekularnom farming proinsulina.

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