J. Agr. Sci. Tech. (2020) Vol. 22(): 1603-1612

Transient Expression of CTB-Exendin Fused Genes in *Nicotiana tabacum* L. via *Agrobacterium tumefaciens*

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ABSTRACT

Exendin-4 is a human Glucagon-Like Peptide-1 (GLP-1) analogue, resistant to DiPeptidyl Peptidase (DPP), which activates the GLP-1 receptor, increases insulin secretion, and improves glycemic control. In this study, Exendin-4 (EX4) was fused to Cholera Toxin B subunit (CTB) and transiently expressed in tobacco leaves. The sequence of the *Ex4* fused to CTB subunit gene, with *Bam*HI and *Sac*I restriction enzymes sites at the beginning of CTB and at the end of *EX4* gene. After codon optimization, the sequence was synthesized and cloned in pUC57 plasmid. The recombinant vectors were transformed into *Escherichia coli* strain DH5α. The pUC57-CTB-EX4 construct was digested with BamHI and SacI restriction enzymes, cloned into pBI121 expression binary vector, and transferred into tobacco leaves through agroinfiltration. Transcription of the *Ex4* fused to cholera toxin B subunit gene in leaves was confirmed by RT-PCR analysis. After agroinfiltration, the protein was extracted from treated leaves, and ELISA test was performed using anti-CTB antibody. The production of recombinant protein was approved by ELISA test in transformed leaves.

Keywords: Agroinfiltration, *CTB-EX4* gene, Restriction enzymes, Tobacco.

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[DOR: 20.1001.1.16807073.2020.22.6.1.7]

INTRODUCTION

Glucagon-Like Peptide-1 (GLP-1) is an incretin hormone secreted by intestinal enteroendocrine L-cells after ingestion of food in response to elevated blood glucose levels. GLP-1 binds to GLP-1 specific receptors on the pancreatic beta cells and improves blood glucose control through mechanisms such as induction of insulin release, increasing the beta cell mass, inhibition of beta-cell apoptosis, promotion of beta-cell neogenesis, reduction of glucagon secretion and gastroparesis (Zappas *et al.*, 2017). GLP-1 has a very short half-life of approximately 2 minutes. Endogenous GLP-1 is rapidly degraded by DiPeptidyl Peptidase-4 (DPP-4) enzyme into biologically inactive form that has lower incretin action of the peptide (Kieffer *et al.*, 1995). Therefore, for the treatment of type 2 diabetes, DPP IV-resistant GLP-1 analogues are needed.

Exendin 4 (EX4), a peptide consisting of 39 amino acids, has been isolated from the saliva of the lizard *Heloderma suspectum* (Eng *et al.*, 1992). EX4 from the lizard saliva is 52% identical at the amino acid level to the mammalian GLP-1 and is encoded from a prohormone distinct from the proglucagon, which encodes the GLP-1 in the intestinal cells (Chen and Drucker, 1997). EX4 binds to the mammalian GLP-1 receptor with the same efficacy as GLP-1 and acts as an effective agonist. EX4 is not rapidly degraded by the DPP IV enzyme and is almost stable (3-4 hours), resulting in provide prolonged incretin effect and contributes to increased insulin secretion due

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to the presence of His-Gly-Glu sequences in N-terminal that is resistant to DPP-IV, whereas the N-terminal sequence of GLP-1 (His-Ala-Glu) is rapidly digested by DPP-IV (Doyle *et al.*, 2003).

Exenatide, a synthetic EX4, is the first approved drug by the Food and Drug Administration that carries out the function of incretin and is used for glycaemic control. The amino acid sequences of Exenatide are identical to the amino acid sequence of EX4. The human trials conducted by using the release form of Exenatide indicate that glycemic control and weight loss is dose dependent with insignificant hypoglycemic effects (Kim *et al.*, 2007). Exenatide is commercially available as a subcutaneous injection, but the requirement for multiple injections decreases patient compliance. Exenatide requires cold storage (between 2 and 8°C) and sterility (DeFronzo *et al.*, 2005). Therefore, the incretin-based therapy of patients with type 2 diabetes requires alternative methods of production and delivery of EX4 to reduce the cost and increase patient compliance.

Oral delivery of recombinant proteins using a strong mucus adjuvant, such as CTB, provides a stronger response (Guo *et al.*, 2014). Cholera toxin B subunit consists of a homopentameric structure, that is approximately 55 kD (11.6 kD monomers) and binds to the GM1-ganglioside on the surface of intestinal epithelial cells and is used as a neurotransmitter due to non-toxicity effect (Androutsellis Theotokis, *et al.*, 2010). CTB is an effective transporter molecule for the antigen that is coupled with it (Guo *et al.*, 2014).

Plants are ideal for expression of therapeutic proteins. The use of plants for the production of valuable recombinant proteins has several advantages for pharmaceutical and industrial uses, such as

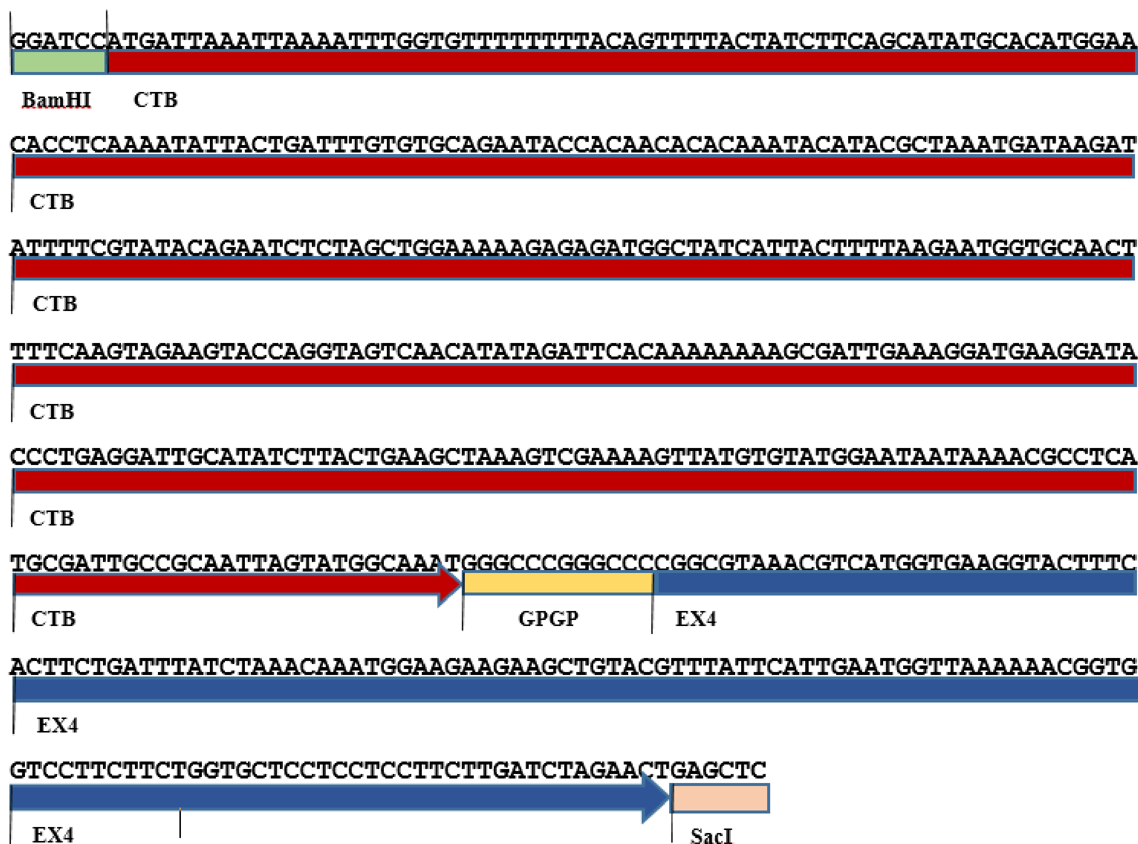

Figure 1. Schematic representation of the synthetic *CTB-EX4* gene.

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safety, scalability, cost-effective productivity, and ease of storage (Fischer *et al.*, 2010). However, the technique suffers from major drawbacks such as low expression level. The expression of the recombinant protein can be increased using many strategies including chloroplast transformation, use of strong promoters, adding untranslated leader sequences, signal peptide, and codon optimization. The Long time required for the generation of transformed plants is another limitation for the production of recombinant proteins (Floss *et al.*, 2001).

The long time required for the generation of transformed plants is another limitation for the production of recombinant proteins in plants. Transient expression in plants has several advantages over the generation of stably transformed transgenic plants such as ease of manipulation, speed, low cost and high yield of proteins, non-contamination with mammalian pathogens, and minimum endotoxin concentration (Hiatt and Pauly, 2006). Transient expression is rapid and protein expression can be obtained in days (Kapila *et al.*, 1997). This makes transient expression suitable for verifying that the gene product is functional before moving on to large-scale production in transgenic plants (Kapila *et al.*, 1997). Stable plant transformation requires considerable investment in time before the expressed proteins can be analyzed. In contrast, transient gene expression systems are rapid, flexible, and straightforward and often use either Agrobacterium or viral vectors.

In this study, we aimed to fuse Exendin 4 (EX4) to Cholera Toxin B subunit (CTB) and transfer it to the tobacco plant by Agrobacterium, and assay transient expression and CTB-EX4 recombinant protein production.

MATERIALS AND METHODS

Construction of *CTB-EX4* Gene

The Cholera Toxin B subunit (CTB) mRNA sequence was obtained from the National Center for Biotechnology Information (NCBI). The *EX4* gene sequence was reconstructed based on Kwon *et al* (2013) article, then, the codon optimize was performed. The Cholera Toxin B subunit (CTB) cDNA sequence was inserted prior to the *EX4* gene sequence. Digestion sites of *Bam*HI and *Sac*I restriction enzymes were introduced into the 5' and 3' ends of the *CTB-EX4* gene, respectively (Figure 1). The Designed *CTB-EX4* fusion gene sequence was synthesized in the Macrogen, Korea. *CTB-EX4* fusion gene was inserted in the pUC57 cloning vector. Thus, the pUC57-*CTB-EX4* plasmid was cloned into *E. coli* strain DH5α. Competent cell was prepared by 100 mM calcium chloride method and bacterial transformation carried out by heat shock method (Sambrook, and Russell 2006). Ampicillin (100 mg. mL^{-1}) -resistant colonies were isolated after overnight incubation at 37°C and pUC57-*CTB-EX4* plasmid was extracted from bacterial cells using alkaline lysis method (Sambrook, and Russell, 2006). To confirm the digestion pattern of the extracted plasmid, *Bam HI* and *Sac I* restriction enzymes were used.

Construction of Gene Cassette

The pBI121 binary vector was used to transfer the *CTB-EX4* gene to the tobacco plant. The pUC57 Plasmid containing the *CTB-EX4* gene and the pBI121 plasmid extracted from *E. coli* were digested by *Bam* HI and *Sac* I restriction enzymes. The *CTB-EX4* gene and the pBI121 vector lacking the *GUS* gene were purified from agarose gel. After removing the *CTB-EX4* gene from pUC57 cloning vector and *GUS* gene from pBI121 binary vector, ligation reaction was performed. The ligation reaction mixture was used to transform *E. coli* strain Top 10 (Sambrook, and Russell, 2006) and kanamycin (100 mg. mL^{-1})-resistant colonies were isolated after overnight incubation at 37°C. Transform colonies were determined using colony PCR and DNA sequencing.

PCR analysis was performed using specific primers. The sequence of forward and reverse

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primers were 5'TGTGCAGAATACCACAACACAC3' and 5'TCAAGAAGGAGGAGGAGCAC3', respectively. PCR was carried out as follow: 94°C 1 minute, 94°C 30 seconds, 58°C 45 seconds, 72°C 30 seconds, 72°C 7 minutes for 28 cycles.

The extracted pBI121-*CTB-EX4* recombinant plasmid by alkaline lysis method was introduced into *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw method (Höfgen, and Willmitzer 1988). Transformed cells were screened by kanamycin (100 mg mL^{-1}) gentamycin (50 mg mL^{-1}) –rifampicin (30 mg mL^{-1}) -resistance and colony PCR after overnight incubation at 28°C.

Transient Expression *via* Agroinfiltration

Agrobacterium tumefaciens strain GV3101 containing pBI121-*CTB-EX4* plasmid was incubated in 2 mL LB medium supplemented with kanamycin, gentamycin, and rifampicin for 48 h and then inoculated into 25 mL LB with appropriate antibiotics. After reaching a cell density of OD600= 1.5, the culture was centrifuged, supernatant was discarded, and the pellet was resuspended in infiltration medium (10 mM $MgCl_2$, 100 μM acetosyringone), adjusted to OD600= 0.5. The suspension was then incubated for 2-3 hours at room temperature and 100 microliters of the bacterial suspension were infiltrated into tobacco (*N. tabacum* cv. Xanthi) leaves using needle-free syringe. Tobacco plants were placed in growth chamber for four days under 23±2°C before being analyzed (Liu *et al.*, 2003).

Detection of *CTB-EX4* Gene in Infiltrated Leaves

RT-PCR (Reverse Transcription-Polymerase Chain Reaction) Assay

Total RNA was extracted from inoculated tobacco leaves with Agrobacterium containing pBI121-*CTB-EX4* construct and non-infiltrated leaf tissue (Mazzara, and James 2000). The cDNA was synthesized after removing DNA and determining the quality and quantity of RNA by Nanodrop Spectrophotometer. RT-PCR test was performed using F-*CTB-EX4* and R-*CTB-EX4* primers.

Enzyme-Linked Immunosorbent Assay (ELISA)

Expression of the *CTB-EX4* gene was further determined by ELISA assay. For the isolation of total proteins from inoculated tobacco leaves and non-inoculated leaf tissue, initially 1 g leaf material was removed and pestled with liquid nitrogen. The powdered leaf material was mixed and homogenized with 2 mL of extraction buffer (aqueous solution with glycerol 1%, and Tris 0.1M pH 7.4). The samples were centrifuged (18,000×g) for 30 minutes at 4°C. The supernatant solution was dispersed in 0.2 μL new tubes and transferred to -40°C for long-term storage (Kim *et al.*, 2007).

Antigen-coating plate (ACP-ELISA) was used to carry out an ELISA test and confirming the presence of recombinant protein (Jiang *et al.*, 2003). After protein extraction from transformed and control plants, the total solution protein concentration was determined using spectrophotometer at 595 nm and Bradford assay. The serum albumin protein was used as concentration reference standard. The same amounts of total soluble proteins extracted from inoculated tobacco leaves with Agrobacterium containing pBI121-*CTB-EX4* construct, Agrobacterium non-containing pBI121-*CTB-EX4* construct (negative control), and non-inoculated leaves (control), with final concentration of 10 $\mu g mL^{-1}$, were mixed with 200 μL of coating buffer and were added to the wells of the ELISA plate. After incubation overnight at 4°C, the ELISA plate was washed 3 times with PBS-T and 3 times with PBS buffer washing buffer. Then, 200

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μL of 1% Bovine Serum Albumin (BSA) (Blocking buffer) was added to each well and placed at 37°C for one hour. After washing the wells with washing buffers, 100 μL of anti-CTB primary antibody (1: 2,000), was added to each well and incubated for 90 minutes at 37°C. The wells were washed by PBST/PBS and then 100 μL of conjugated antibody (anti rabbit IgG 1:1,500) was added to the wells and incubated for 60 minutes at 37°C. Wells were developed with TMB substrate; the color reaction was stopped by 2/5N H_2SO_4 and the absorbance was read by an ELISA reader at 450 nm.

RESULTS

The *CTB-EX4* fusion gene was synthesized and resequenced for confirmation. *CTB-EX4* fusion was cloned in the pUC57 vector and transformed to *E. coli* strain DH5α. Presence of pUC57 in *E. coli* was confirmed by colony PCR and digestion with *Bam*HI and *Sac*I enzymes, which produced 537 bp fragment for the *CTB-EX4* gene.

The pBI121 vector was digested by *Bam*HI and *Sac*I enzymes (Figure 2-a). Digestions by these enzymes eliminate the GUS coding region, which is located after CaMV 35S promoter in pBI121 vector. The digested vector was gel purified and ligated with *CTB-EX4* fusion gene. After ligation and transformation, the obtained recombinant colonies from LB medium containing 100 mg.mL^{-1}of kanamycin were evaluated by colony PCR reaction (Using F-*CTB-EX4* and R-*CTB-EX4* primers) and sequencing of *CTB-EX4* gene, then, clones containing plasmid pBI121-*CTB-EX4* were screened. An expected band size of 429 bp was observed for the PCR product obtained from extracted plasmids from positive colonies (Figure 2-b). The result of pairwise alignment between *CTB-EX4* gene and the sequenced obtained from recombinant constructs, confirmed *CTB-EX4* insertion in pBI121 vector.

pBI121-*CTB-EX4* construct was transformed to *Agrobacterium* strain Gv3101 using freeze-thaw method. Recombinant colonies grown on LB medium containing kanamycin, rifampicin, and gentamicin antibiotics were selected. The presence of pBI121-*CTB-EX4* was confirmed by colony PCR using F-*CTB-EX4* and R-*CTB-EX4* primers in Gv310.

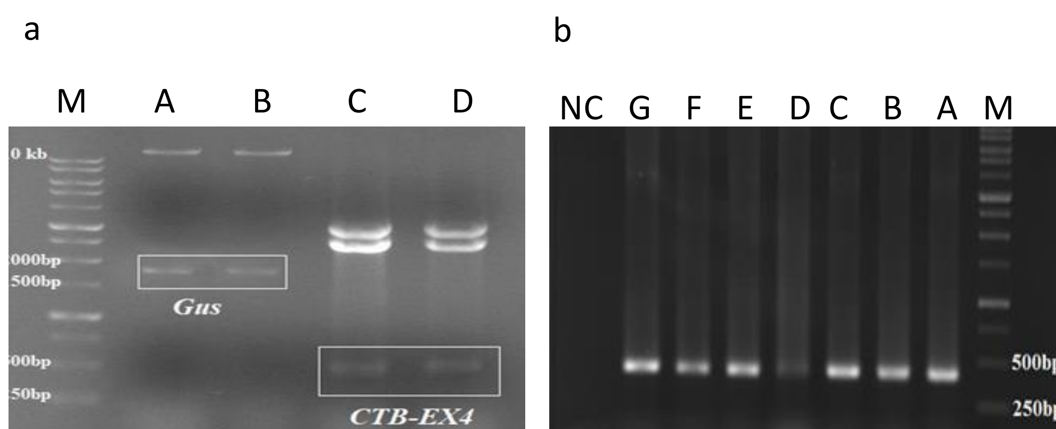

Figure 2. The result of enzymatic digestion pUC57-CTB-EX4 and pBI121 plasmid plasmids on 1% agarose gel (a), PCR analysis for detection of CTB-EX4 gene in transformed *A. tumefaciens*. (M): 1 Kb Size marker. (A, B): Digested pBI121plasmid, (C, D): Digested pUC57-CTB-EX4 plasmid. b) Colony PCR of CTB-EX4 in pBI121 in *A. tumefaciens*. M): 1 Kb Size marker. (A, B, C, D, E, F, G): Agrobacterium recombinant colony (NC): Negative Control.

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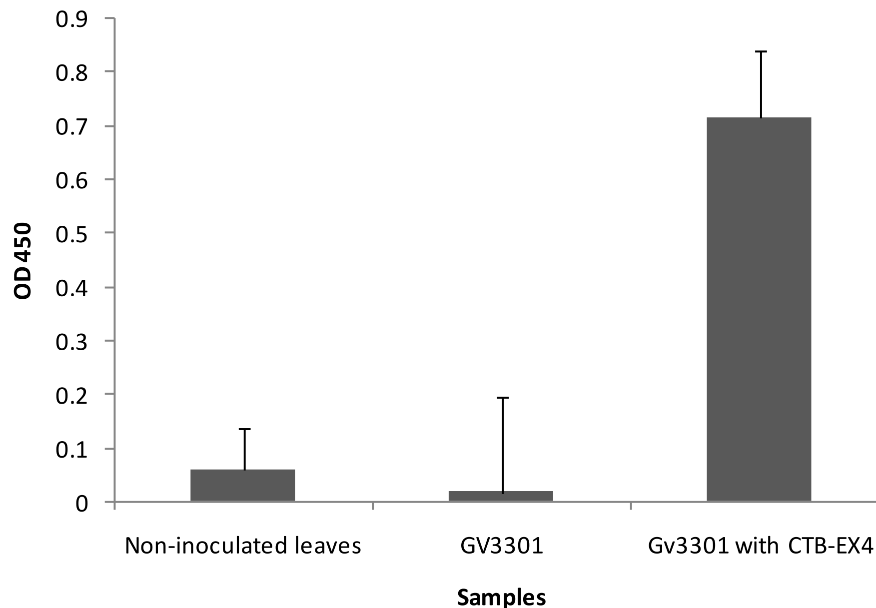

Figure 4. ELISA analysis of CTB-EX4 protein in agro-infiltrated tobacco leaves at 4 days after agro-infiltration at 450 nm wavelength. Non-inoculated leaves (negative control). (Gv3101): Protein extracts from inoculated tobacco leaves with Agrobacterium strain without *CTB-EX4* gene, (Gv3101 with CTB-EX4): Protein extracts obtained from inoculated tobacco leaves with Agrobacterium strain Gv3101 containing CTB-EX4 gene. The values are the average OD derived from five replicates, with standard deviations.

Analyses of Transient Expression by RT PCR and ELISA Assay

Tobacco leaves were transformed via agroinfiltration (Figure 3). Expression of CTB-EX4 was evaluated at transcription level using RT PCR. Results showed that the foreign gene was transcribed in infiltrated leaves. Expected band was observed for the mRNA sample of transformed leaves, but not for the wild type plant (Figure 4).

Translation of *CTB-EX4* gene was evaluated by ELISA assay. Results confirmed expression of the foreign gene at translation level. As shown in Figure 4, the average Optical Density (OD) reading of the plant samples expressing the CTB-EX4 recombinant protein was significantly greater than that of both negative controls.

DISCUSSION

Exenatide is an important drug in the treatment of diabetes and its incretin activity increases insulin secretion and regulates glucose blood in mammals (Goldberg and Gomez Orellana, 2003). At present, the daily cost of an injectable Exenatide drug that should be consumed twice a day is more than a few thousand dollars a year, which is not possible in developing countries with a high population and income less than $2 a day (Bond, 2006). Therefore, Exenatide analogue production (Exendin 4) in a plant expression system and its oral delivery can be an appropriate solution to the problem.

In this study, in addition to the GPGP sequence, the furin digestion site was placed between *CTB* and *EX4* gene. At oral delivery of CTB-EX4 recombinant protein, after binding the CTB protein to GM1

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receptors on the intestinal epithelium, the furin enzyme releases EX4 into the blood stream by digesting the *CTB-EX4* gene at RRKR digestion site (arginine, arginine, lysine, arginine) (Kwon *et al.*, 2013).

The pBI121 vector used to clone and express *CTB-EX4* gene is a binary vector for gene expression in plants, containing two left and right borders: a kanamycin resistance gene as a selective agent to isolate bacteria that have taken up genes and a *Gus* reporter gene (*β-glucuronidase*) for the analysis of promoter activity, a CaMV 35S promoter for *CTB-EX4* gene expression in transgenic plants, a NOS terminator, and also restriction sites within an MCS region (Kumar *et al.*, 2003). To insert the *CTB-EX4* gene in the pBI121 vector, the pBI121 vector and the desired gene were digested with the same two enzymes.

Low level of gene expression in stable transformation experiments is a major obstacle for production of recombinant proteins in green plants. In addition, generation of stable transgenic line required long times. An approach to address these limitations is application of transient gene expression assays (Gleba *et al.*, 2007). In the present study, tobacco leaves were transiently transformed with a *CTB-EX4* chimeric gene via agroinfiltration. The method has been reported as an efficient and rapid procedure for transient gene expression in plants. As can be seen from ELISA assay, the protein sample obtained from transformed leaves was bound to the anti-CTB antibody in ELISA Microplates (96-well), and comparison of ODs obtained from the ELISA graph showed a significant difference between the negative controls and the treatments related to using Agrobacterium containing pBI121-*CTB-EX4* construct**.** Various factors can influence transient gene expression, including leaf position, OD of the infiltration, and temperature after agro-infiltration (Leckie, and Stewart 2011). Further enhancement of the transgene expression might be achieved by optimizing these factors. To enhance the expression of CTB-EX4 recombinant protein, chloroplast transformation or attaching chloroplast targeting signal can be efficient (Jobling, *et al.*). Also, to enhance the expression of the *CTB-EX4* gene, the Kozak consensus sequence (ACCAUGG) can be placed at the 5' end of the *CTB-EX4* genes. In addition, for convenient purification, the his-tag sequence can be used (Dhar *et al.*, 2014).

In summary, the results showed that a good level of CTB-EX4 expression was achieved in our experiment in only 4 days after agroinfiltration. These findings confirm that transient gene expression, as a rapid and efficient production system, could be used to produce the Ex4 protein for the treatment of type 2 diabetes.

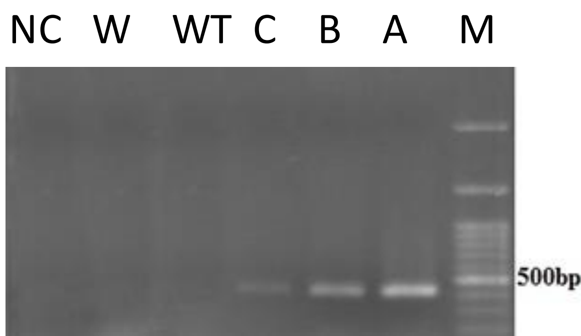

Figure 3. RT-PCR products on 1% agarose gel. (M): 100 bp Size marker; (A, B, C): Inoculated Leaves with *Agrobacterium* containing pBI121-CTB-EX4 recombinant vector; (W): Inoculated Leaves with *Agrobacterium* containing pBI121 vector, (WT): Wild Type plant (non-inoculated leaves, negative control).

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ACKNOWLEDGEMENTS

We would like to thank Dr. Hosein Honari for his kind gift of the anti-CTB primary antibody. We also gratefully acknowledge the financial support from the Iran National Science Foundation (INSF) [research project 95849683] and Faculty of Agriculture of the University of Kurdistan, Iran.

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بیان موقت ژن اکسندین-٤ متصل به زیر واحدBسم وبا (CTB) توسط آگروباکتریوم در تنباکو (*Nicotiana tabacum* L.)

ش. کوکه ای، و ب. بهرام نژاد

چکیده

اکسندین-۴ آنالوگ GLP-1 انسانی می‌باشد که مقاوم به آنزیم دی پپتیدیل پپتیداز IV است و ترشح انسولین را افزایش داده که منجر به بهبود کنترل قند خون می شود. در این مطالعه، اکسندین-۴ جداسازی شده از بزاق مارمولک، که آگونیست GLP-1 می‌باشد، به‌عنوان یک پروتئین متصل شده به زیرواحد B سم وبا، به‌طور موقت در گیاه تنباکو بیان شد. ابتدا توالی ژن اکسندین-۴ متصل به زیرواحد B سم وبا طراحی و جایگاه‌های برش آنزیمی *Bam* HI و*Sac*I به‌ترتیب در ابتدا زیرواحد B سم وبا و در انتهای توالی ژن اکسندین-۴ قرار داده شد و سپس توالی طراحی شده سنتز گردید. ژن اکسندین-۴ متصل به زیرواحد B سم وبا همسانه‌سازی شده در داخل ناقل کلونینگ PUC57، به باکتری اشرشیاکلی منتقل گردید. سپس پلاسمید pUC57-*CTB-EX4* استخراج شده از باکتری اشرشیاکلی، با استفاده از

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آنزیم‌های محدودکننده *Bam* HI و*Sac*I برش داده شد و در ناقل دوتایی بیانی pBI121 همسانه‌سازی گردید و از طریق آگروواینفیلتراسیون به برگ گیاه تنباکو انتقال یافت. رونویسی ژن اکسندین-۴ متصل به زیرواحد B سم وبا، به‌روش RT-PCR در برگ گیاه تنباکو تایید گردید. از برگ‌های آگروواینفیلتره شده، پروتئین کل استخراج گردید و با استفاده از آنتی بادی ضد زیرواحد B سم وبا، آزمون الایزا صورت گرفت و تولید پروتئین نوترکیب تایید گردید.

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