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Production of Lettuce Edible Vaccine for Cholera Disease Using Chloroplast Genetic Engineering.

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ABSTRACT

Lettuce is one of the most important edible plant worldwide. At the time that lettuce is the candidate plant to carry the foreign vaccine gene for human. The B subunits of toxin of *Vibrio cholerae* (CTB) are candidate vaccine antigens. This research was conduct to express *CTB* gene in lettuce chloroplast. Genes required in this study were obtained by polymerase chain reaction (PCR) technique using specific forward and reverse primers, and these genes were *CTB*, *BADH*, *prrn* promoter and many other regulatory genes. Some of these genes were isolated from their hosts and some were obtained from previous work available at Daniell laboratory. All these genes beside many techniques for ligation, extension, sequencing, orientation confirmation were used to construct the cassette vector pLS-BADH-LS-CTB which carries the gene of interest. In this work the *CTB* gene with *BADH* gene were transferred to the chloroplast of lettuce plant and selection of transgenic plant was performed on the MS medium containing BA and NaCl without any antibiotic selectable marker. Integration of an unmodified CTB-coding sequence into chloroplast genomes (up to 1000 copies per cell) resulted in the accumulation of up to 6.2% of total soluble lettuce leaves protein as functional oligomers (620-fold higher expression levels than that of the unmodified CTB gene expressed via the nuclear genome). PCR and Southern blot analyses confirmed stable integration of the *CTB* gene and *BADH* gene into the chloroplast genome in addition to the integration in the right orientation and in specific region between *trnaI**trnA*.Western blot analysis showed that the chloroplast synthesized CTB assembled into oligomers and were antigenically identical with purified native CTB.

Introduction

There is currently much enthusiasm for the potential of genetically engineered plants through production of edible vaccines to help controlling human and animal diseases. Plants expressing bacterial and viral antigens as nuclear transgenes are capable of triggering immune responses when the transgenic tissues are administered orally. Indeed, it has been suggested that plant cells containing an oral vaccine may actually potentiate vaccine activity by protecting against premature digestion of the antigen [1]. Despite this promise, transgenes expressed via the nucleus often yield insufficient antigen levels, reported as total soluble protein (TSP) or fresh weight (FW): B subunits of enterotoxigenic *Escherichia coli* (0.01% TSP), hepatitis B virus envelope surface protein (0.01%TSP), human cytomegalovirus glycoprotein B (0.02%TSP), and transmissible gastroenteritis coronavirus glycoprotein S (0.06%TSP). Therefore, one ever present mission is to increase the level of transgene expression within transgenic plants [2].

Cholera toxin B subunit (CTB) is a candidate oral subunit vaccine against cholera, a disease that causes acute watery diarrhea by colonizing the small intestine and producing the enterotoxin, cholera toxin (CT). Increased expression levels of several proteins have been attained by expressing foreign proteins in chloroplasts of higher plants. Recently, human somatotropin (7%TSP) and antimicrobial peptides (21% TSP)22 have been expressed in transgenic chloroplasts. The accumulation levels of the Bt

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Cry2Aa2 operon in tobacco chloroplasts were as high as 46.1% of the total soluble plant protein [3]. Besides the ability to express polycistrons, another advantage of chloroplast transformation is the lack of recombinant protein expression in pollen of chloroplast transgenic plants. Absence of chloroplast DNA in the pollen of most crops reduces pollen-mediated outcross of transgenes. Also, stable incorporation of the *CTB* gene into spacer regions between functional genes of the chloroplast genome by homologous recombination eliminates the "position effect" frequently observed in nuclear transgenic plants. Lack ofgene silencing in chloroplasts should allow uniform expression levels in different transgenic lines. Integration of the transgene into chloroplast genomes should result in a high level of *CTB* gene expression, since each plant cell contains up to 1000 copies of the plastid genome. Similar to the endoplasmic reticulum, the production of CTB in chloroplasts allows formation of disulfide bridges, which are necessary for the correct

Materials And Methods

Gene isolation:- Various primers were used throughout this study to isolate the genes of interest and to investigate the transgenic plant. All primers were designed depending on the sequence available on NCBI web site and the primers were carefully designed using available software (primer select module of DNASTAR lasergene program. All these primers have melting temperature (Tm) ranging between (60-65°C), The annealing temperature (Ta) for each primer pair was generally calculated as 5°C lower than the estimated melting temperature. The primers used were:

(CTB)TATGGATCCATGACACCTC AAATATTACT,GGCGAATTCATATCTTAATTTG CCATAC for (BADH) CACTCTGCTGGGCCGACACTGACAC,CACTAGC CGACCTTGACCCCTGTT for (Prrn) ATCGATGAGCCTGATTATCCTAAG,CAGCAGGT AGACAAAGCGGATTC

Vector Construction:- [1,5,6]

The pUC-based L. sativa long flanking plasmid (pLS-LF) was used tointegrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome inverted repeat region [3]. A transformation cassette for the generation of transplastomic *L. sativa* plants that express CTB-BADH from the T7 gene translational control region was transferred to pLS-LF from pZERO (Invitrogen, Carlsbad, CA). The cassette included the following published *N. tabacum* plastid regulatory sequence elements: ribosomal operon promoter (Prrn), psbA 3' UTRs. The BADH gene was included conferring salt tolerance and was expressed via a GGAGG ribosome binding site. All digest products (vectors and inserts) were separated by electrophoresis through 0.8% agarose-TAE (400 mM Trisacetate, 10 mM EDTA) gels containing 1ug mL-1 ethidium bromide. Gel fragments were isolated using a sterile blade under UV illumination and DNA was eluted from gel fragments using the QIAquick gel extraction kit . Plasmid products of T4 ligase mediated reactions were transformed into *E.* coli according to standard protocols [7]. The expression cassette was digested with SnaBI. pLS-LF was digested with PvuII and treated with alkaline phosphatase prior to ligation with the SnaB1 digested cassette. Recovered plasmids were digested with restriction enzymes to determine correct orientation of the inserted cassette in pLS-LF. Nucleotide sequence of the intermediate plasmid was confirmed.

Transformation protocol :- For transformation of plant with any gene using gene gun technology, gold particle must be prepared firstly, then the particles are bombarded to plant tissue. Different plant has different parameters to transformed using gene gun ,but in general, plant tissue must be healthy and grown aseptically and all techniques must be done in aseptic airflow laminar hood. Transformation of lettuce leaves were done according to Kumar, *et al* protocol in 2004[8].

Confirmation of transgenic plant:-After 2 days of incubation in the dark culture room, the bombarded leaves were transferred to hood and cultured on selective medium. Each of the bombarded leaf was cut into small (5 mm2) pieces and place the adaxial side (bombarded side) touching LRM selection medium containing 35mM betaine aldehyde . Each Petri dish was wrapped with parafilm and kept in the culture room under appropriate growth conditions. Within 3–5 weeks of culture, putative transformants will appear. Again the leaves of the primary regenerated PCR-positive putative transplastomic shoot were cut into small pieces (2 mm2) and subject to a second round of selection on fresh LRM selection medium to achieve homoplasmy. The regenerated

shoots after second round of selection were separated and transferred to LRM selection medium containing 35mM betaine aldehyde for third round of selection . PCR technique was used to screen transgenic plants and to distinguish transplastomic plants from the mutants. The integration of transgene into sitespecific chloroplast genome is determined by using sets of primers, one of which anneals to the native chloroplast genome beyond the flanking sequence and the other anneals to the transgene cassette . No PCR amplification will be observed in mutants and nuclear transgenic plants with these primers. To confirm the site-specific integration of transgene cassette into chloroplast genome, perform the PCR using primer pairs 16SFCAGCAGCCGCGGTAATACAGAGGA and BADH reverse primer.

Southern and Western Blot Analyses:- Initially, few copies of the chloroplast genomes receive transgene cassette and after several rounds of selection, untransformed genome copies are replaced by transgenic copies, leading to homoplasmy. This can be ascertained by Southern blot analysis of the total DNA isolated from the plants after third round of selection.

Total plant DNA extracted from transplastomic leaves was digested with Sma1 and probed with DIG chloroplast flanking sequences. The standard protocol of southern blot were applied depending on Verma and Daniell [5] Western blot were done to check the expression of CTB protein in transgenic plant and the standard protocol reviewed by Singh *et al* [9].

Result and Discussion

Gene Isolation:-One of the aims of this study is to produce an edible vaccine against cholera disease. To achieve this aim, a method to isolate the *CTB* gene from *Vibrio cholerae* obtained from the infectious disease laboratory at Burnett School for Biomedical Science was applied. Primers for isolation of *CTB* gene were designed according to primer-3 program available at NCBI web site, and a molecular weight of *CTB* gene of about 375 bp was obtained(fig 1 a), while the resulted bands were eluted using standard protocol . Cholera toxin B subunit (CTB) is the pentameric non-toxic portion of cholera toxin (CT), responsible for the holotoxin binding to the GM1 ganglioside receptor present on most nucleated cells . When conjugated to autoantigens, the CTB dramatically increases their tolerogenic potential after oral administration[10].

In this study, BADH gene was isolated from a previous work of Kumar *et al.* [8] in Professor Dniell laboratory at University of Central Florida using BADH forward and reverse primers according to the sequence of BADH gene isolated from spinach. Results of electrophoresis showed that the gene could be isolated in a single band with a molecular weight of about 1470 bp (fig 1 b). The gene then was eluted for further experiments to construct the vector. Higher plants from several families (e.g., Chenopodiaceae, Poaceae, Asteraceae) accumulate the quaternary ammonium compound betaine in response to salt stress or water deficit. Much evidence indicates that in plants and in other organisms, betaine acts as a nontoxic or protective cytoplasmic osmolyte, allowing normal metabolic function to continue in cells at low solute potential [11].

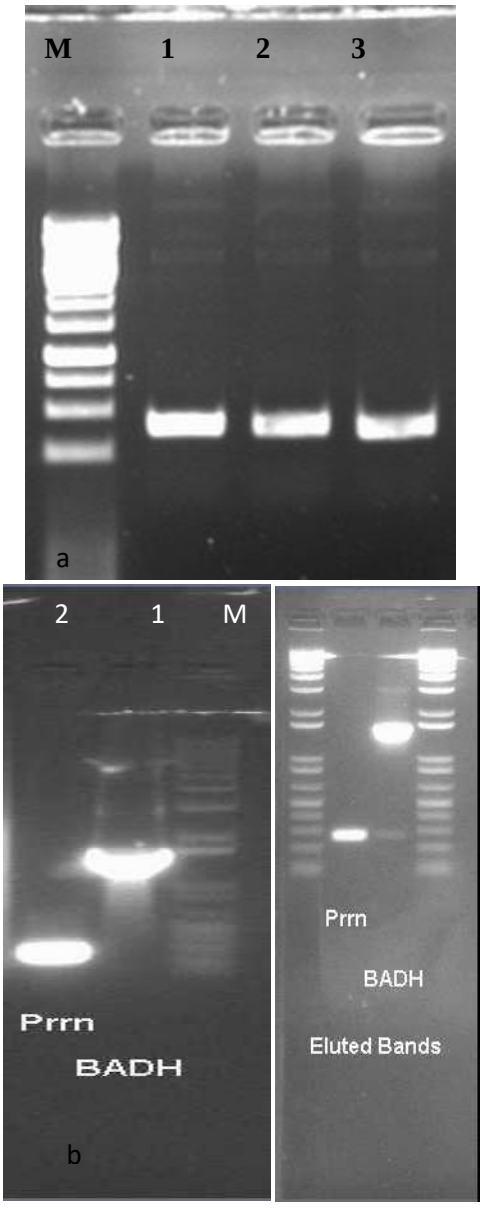


Figure 1:- Isolation of gene by PCR (a) CTB gene from *V. cholerae* (b) BADH and prrn from previous vector

Vector construction:- After the required genes were isolated, other steps of the project were started such as the construction of shuttle vector and cassette vector containing CTB and BADH, prrn promoter, all regulatory genes, and the specific restriction site. To achieve such steps, precise work regarding many PCR processes, restriction, ligation, sequencing, and transformation in *E. coli* were performed. Vector construction started with the ligation of BADH gene with prrn promoter previously isolated using BADH forward and prrn reserve primers formally designed in their isolation. The product was about 1700 bp DNA segment eluted and used to complete the shuttle vector (2 a). Trps16(rbcl) 150 bp was amplified from genomic DNA of lettuce and used as transcription termination and enhancing the translation, and was ligated with prrn, BADH. All these products were ligated in pBs vector available at Professor Daniell laboratory. This vector contains ampicillin resistance gene, and therefore, it was able to transform *E. coli* with this vector to propagate new vector and to ensure the right work .Figure (2 b) shows that the pUC-based *L. sativa* long flanking plasmid (pLS-LS) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome [3]. The pLS-LS vector was modified to include the BADH gene for selection of transplastomic lines. Expression of BADH is driven by the *L. sativa* endogenous Prrn and a GGAGG ribosome binding site. The BADH transcript is stabilized by inclusion of the endogenous 3' UTR of rbcL [6]. The CTB gene construct was inserted into the pLSLS vector as previously established by Professor Daniell laboratory [5,1] under the control of the light regulated psbA region located within the 5' UTR. All previous vectors contained the aadA gene which confers resistance to spectinomycin and were driven by the upstream prrn promoter but in present study BADH gene was used instead of aadA as selectable marker to establish the transformed plants. Chloroplast vectors included native lettuce DNA flanking regions (trnI/trnA) in order to facilitate homologous recombination [1].

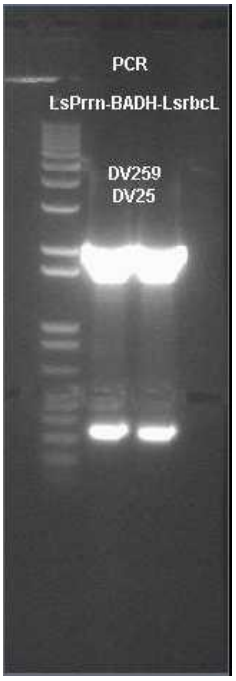
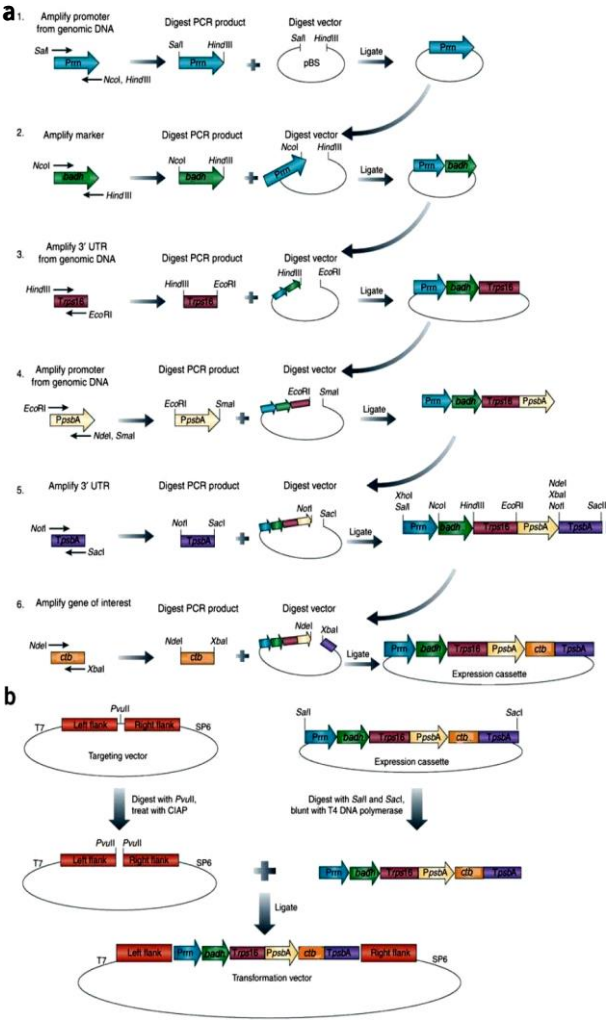


Figure 2:- (a) Gel electrophoresis of PCR product for gene ligation of BADH,prrn (b) complete details for cassette vector construction



The complete physical map for the cassette vector is show in the following figure (3)

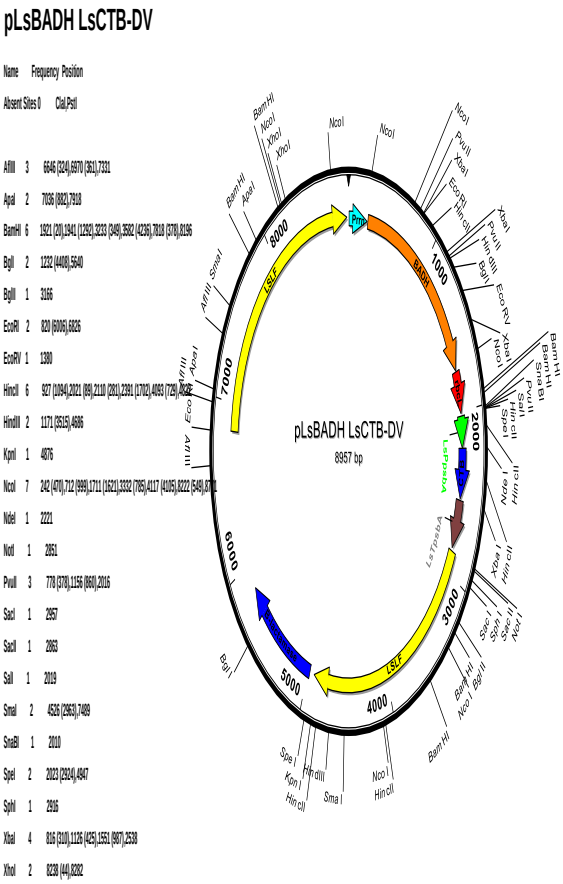


Figure 3: physical map for cassette vector carry BADH, CTB genes

Screening of transgenic plant:-

Many plantlets appeared after various incubation period on MS medium containing selectable agents. These plantlets should be tested to ensure transformation. PCR technique was used to confirm the transgenic plant using the same primer used for *BADH* isolation, and to confirm plastid integration, 16SF primer which anneal only to native plastid DNA with other primer inside the cassette vector. (Figure 4 a) shows different plantlets that appeared after 14-25 days of incubation on a medium containing NaCl and BA as selectable agents. Results showed that most of regenerated plants were transgenic and contain BADH gene (4b). Testing of BADH was carried out by the same primer to isolate BADH.

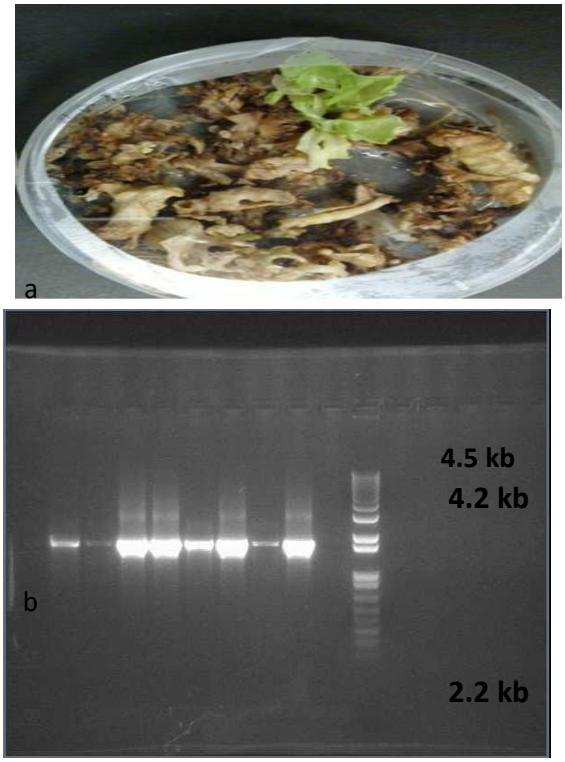


Figure 4:-(a) expected transgenic plant in selective medium (b) gel electrophoresis of PCR product for DNA isolated from expected transgenic plant by BADH primers.

Confirmation of chloroplast integration of cassette vector was conducted by PCR but with primers annealed only to chloroplast native genome which was 16SF primer as shown in the chapter two. This primer annealed to the region out of flanking sequence and gave a product more than 2000 pb since the flanking sequence was 2000 bp from each side. Primers used to confirm chloroplast integration of cassette vector were 16SF and primer inside BADH gene, according the primers used the fragment resulted was 2900 bp (figure 5). Results showed many regenerated plants with chloroplast Southern blot analysis was performed. Total DNA extracted from plants in the third round of selection was digested with enzyme Sma1. The digested DNA on agarose gel was hybridized with a chloroplast flanking sequence probe (0.8kb). As shown in the figure (figure 6), Wild type plants generated 4.2 kb fragment and transgenic

plants generated 4.5 and 2.9 kb fragments. integration.

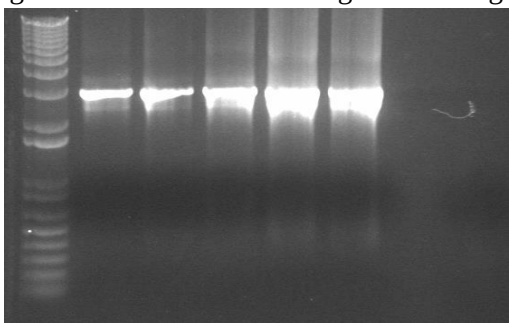


Figure 5:- PCR product for isolated DNA from transgenic plant using 16SF primer with BADH primer

All transgenic lines appeared homoplasmic (within the levels of detection) which means that all chloroplasts in the plant contained the transgene BADH-CTB.

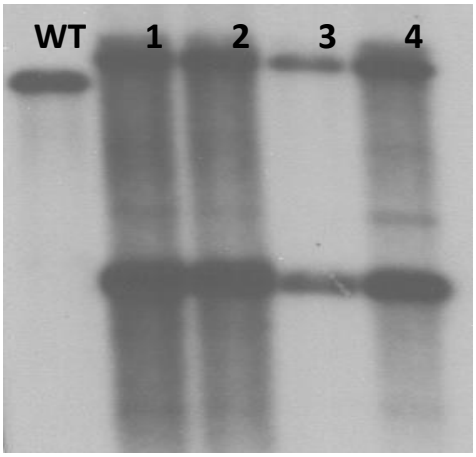
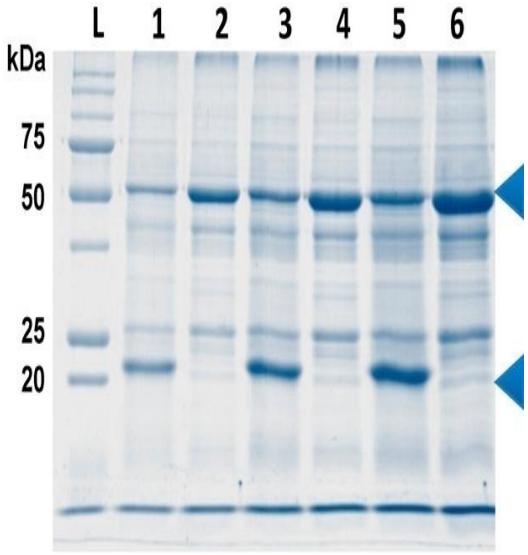


Figure 6:- Southern blot analysis with flanking sequence probe. Lane WT Wild-type showing 4.2 kb fragment, Lane 1 - 4 Transgenic plants showing 4.5 and 2.2 kb hybridizing fragments.

The tissue culture medium triggers cell division, yielding meristematic cells with 10 to 14 proplastids, each of which carries only one or two nucleoids. Reduction of plastid number from 100 to 10 to 14 greatly accelerates plastid sorting during cell division, during which plastids carrying the T-ptDNA are dividing at a faster rate. Plastids carrying only the wild-type ptDNA are ultimately lost by dilution during cell division [12].

Western blot analysis was performed to investigate the expression of the fusion protein CTB-BADH in transgenic lettuce chloroplasts, using anti-CTB antibody. The protein was extracted from the lettuce leaves as described standard protocol, its concentration was calculated using standard curve made by bovine serum albumin (BSA) using Bradford method (Bradford 1976). Different amounts of total protein were loaded on 12% polyacrylamide gel to show the protein bands, and the resulted bands indicated a new protein band in transformant plant with about 22 KDa (figure 7). In comparison with wild type plant, this is an indicator for expression on monomer of CTB protein which has the corresponding molecular mass.



Figure(3-25) SDS-PAGE stained with Coomassie Brilliant Blue. Lanes 1, 3, and 5 laoded with 10, 20, and 30 ug respectively, of total soluble protein from transgenic leaf, lanes 2, 4, and 6, corresponding amounts of wild type protein extract, lane L, molecular mass standards. Arrow heads indicate positions of CTB (22 kD) and Rubisco (53 kD).

The percentage of CTB expressed was as a percent of total soluble protein calculated using the Bradford assay i.e. the CTB percent is inversely proportional to the TSP values. The CTB expression levels reached a maximum of 6.3% of the total soluble protein in the old leaves when compared to 2.6% TSP in young leaves, and 5.2% TSP in mature leaves .Maximum CTB expression was observed in the old leaves when compared to the young and mature leaves.

Besides the ability to express polycistrons, another advantage of chloroplast transformation is the lack of recombinant protein expression in pollen of chloroplast transgenic plants. Absence of chloroplast DNA in the pollen of most crops reduces pollen-mediated outcross of transgenes. Also, stable incorporation of the CTB gene into spacer regions between functional genes of the chloroplast genome by

homologous recombination eliminates the (position effect) frequently observed in nuclear transgenic plants. Lack of gene silencing in chloroplasts should allow uniform expression levels in different transgenic lines. Integration of the transgene into chloroplast genomes should result in a high level of CTB gene expression, since each plant cell contains up to 1000 copies of the plastid genomes [13]. Similar to the endoplasmic reticulum, the production of CTB in chloroplasts allows formation of disulfide bridges, which are necessary for the correct folding and assembly of the CTB pentamer [14].

This is the pioneer study to express CTB antigen in an edible plant via chloroplast genetic engineering, and antibiotics selectable markers were replaced by safe selectable marker (BADH) expressing salt and drought resistance.

Also chloroplast genetic engineering is proved to be sufficient in expressing new foreign protein (quantity and quality) in plants.

Acknowledgment

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14- DeGray, G.; Rajasekaran, K.; Smith, F.; Sanford, J. and Daniell, H. (2001). Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol., 127:852– 862. folding and assembly of the CTB pentamer 4. We here expressed CTB in plastids of lettuce using BADH gene for salt tolerance as selectable marker gene.

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انتاج لقاح الخس المأكول لمرض الكوليرا باستخدام الهندسة الوراثية للبلاستيدات الخضراء

احمد عبد الجبار سليمان كاظم م. ابراهيم عبد الواحد باقر الشيباني

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الخلاصة:-

يعد نبات الخس واحد من النباتات المأكولة المهمة في العالم , ويتعبر ايضا نبات مرشح لحمل الجينات الغريبة الى الانسان. يعد الجزءB من سم بكتريا *Vibrio cholera* من الانتجينات المرشحة للعل كلقاح للانسان. صممت هذه الدراسة لنقل جين المسؤول عن انتاج السم B الى البلاستيدات الخضراء لنبات الخس. عزلت الجينات المطلوبة لعمل هذه الدراسة باستخدام تقنية تفاعل استطالة السلسلة PCR والذي استخدم لعزل الجينات التنظيمية مع CTB, BADH and Prrn وقد تم عزل هذه الجينات من اماكن تواجدها باستخدام بادئات خاصة وقسم منها تم عزلها من نواقل كلونة مصممة سابقا لهذا الغرض في مختبرات الدكتور دانيل في جامعة سينترال فلوريدا. استخدمت عدد من التقنيات بجانب تفاعل استطالة السلسلة للحصول على ناقل كلونة يحمل جين السم B وهذا الناقل هو pLS-BADH-LS-CTB , واستخدمت طريقة حقن الجينات باستخدام مسدس الجينات لحقن الجين الى البلاستيدة الخضراء لورقة الخس وتم الانتخاب الوراثي للنباتات المهندسة وراثيا على اساس تحملها للملوحة ومادة البيتايين الديهايد بدلا عن مقاومة المضادات الحيوية وهذا ما جعل هذا العمل اول عمل يتم لانتخاب الكائنات المهندسة وراثيا على اساس تحمل الملوحة. حملت النباتات المهندسة وراثيا جين انتاج لقاح الكوليرا في البلاستيدات الخضراء البالغ عددها حوالي 1000 بلاستيدة وكانت نسبة التعبير الجيني حوالي 6.2% من البروتين الكلية المعزول من النبات. استخدمت تقنية تفاعل استطالة السلسلة وتقنية وصمة ساوثرن لتاكيد المكان الصحيح لدخول الجينات في حين استخدمت تقنية وصمة ويسترن للتاكدي على انتاجية النبات ودقة التعبير الجيني.