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Original Article

Recombinant Production of a Chimeric Protein Containing StxB, Eae, Cfab, and LTB Proteins in Hairy Roots of *Nicotiana tabaccum*

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Abstract

Introduction: The technology of recombinant protein production has revolutionized biological, pharmaceutical, and medical researches. Plants are appropriate systems for the production of recombinant proteins. In the present study, a chimeric gene encoding a chimeric protein containing StxB, Eae, CfaB, and LTB as a candidate vaccine against enterotoxigenic and enterohemorrhagic *E. coli*, was transferred to the hairy root of the tobacco plant and its expression was analyzed.

Materials and Methods: For this aim, the plant-based codon-optimized gene, which was synthetically cloned in pBI121 binary vector, was transferred into *Agrobacterium rhizogenes* competent cells. *A. rhizogenes* cells containing pBI121+*secl* were transferred into sterile *Nicotiana tabaccum* plants by direct and indirect methods. Finally, the expression of recombinant protein was analyzed by the ELISA method.

Results: The transformation of *A. rhizogenes* cells and Tobacco plants was confirmed by PCR amplification of a fragment of the chimeric gene with specific primers. SDS-PAGE and ELISA analysis showed the successful expression of the recombinant chimeric protein.

Conclusions: In conclusion, it was shown that the hairy roots of tobacco plant is a proper expression system for the recombinant production of SECL protein. The protein can be used for mice's immunization against enterotoxigenic and enterohemorrhagic *E. coli* strains.

Keywords: Protein Expression, Hairy Roots, *Nicotiana tabaccum*, Vaccine Candidate

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Introduction

Recombinant production of proteins, including medicinal and pharmaceutical ones, had a great impact in nearly all industries.[1,2] In the case of vaccinology, this technology in combination with the relatively young field of immune informatics has made it possible to design and produce completely new antigens for immunization against one or more pathogenic bacteria.[3,4]

The life on the earth is truly dependent on plants. Besides many advantages of these organisms, they have been exploited as potent protein production factories.[5] High expression capacity, well-understood manufacturing practices, low cost, safety, a high level of scalability and the existence of infrastructures are some of the desirable characteristics of the plants.[6] Recombinant proteins have had expressed in different parts of plants, including leaves, stem, seeds, etc.[7] Since plants can be used orally, they are so appropriate for the production of recombinant vaccines against pathogens, especially those which infect hosts via mucosal routs, including enteropathogenic bacteria, such as Enterotoxigenic *E. coli*, *Vibrio cholerae*, pathogenic species of *Shigella*, etc. In this regard, many plant-based recombinant proteins have been designed and evaluated against these pathogens.[8-10] Although recombinant proteins are successfully produced in transgenic plants, however, regulatory issues have always been a major concern in this regard. For this reason, cultures of differentiated organs, like hairy roots, or plant cell cultures are proper alternatives to whole transgenic plants.[11]

Hairy roots have special characteristics, such as a fast growth rate, easy cultivation, no need for complex culture media, being highly branched, etc. Since these structures are differentiated, they are stable, both phenotypically and genetically.[12,13] They can be derived from a variety of plant species and they often have the same metabolites of the origin plant. Like plant cells, hairy roots have the post-translational protein modification systems. The purification of the expressed recombinant proteins in hairy roots is relatively easy because most of the time, they are secreted in the culture media and it is so easy to purify the desired protein.[11,14]

In this study, a candidate recombinant vaccine called SECL

[comprising StxB (B domain of Shiga-like toxin), Eae (*E. coli* attaching and effacing), Cfab (colonization factors antigen b), and LTB (heat-labile enterotoxin B subunit)] was expressed in hairy roots of Tobacco plants for immunogenicity evaluation against enterotoxigenic *E. coli*.

Materials and Methods

Enzymes, Chemicals, Kits, Antibiotics, Culture Media and Plant Seeds

EcoRI and HindIII restriction endonuclease enzymes were prepared from Thermo Fischer Scientific (USA). Pfu and Taq DNA polymerases along with materials needed for polymerase chain reaction (PCR) were purchased from SinaClone (Iran). All chemicals were prepared from Merck Company (Germany) and were of molecular biology grade purity. Anti-mouse conjugated antibody was prepared from Sigma Company (Germany). ELISA plates were prepared from NUNC Company (Denmark). Antibiotics were purchased as follows: Ampicillin from Roch (Germany), Kanamycin from Applicum (Germany), Cefotaxime from Jaber Ebne Hayyan Pharmaceutical Company (Iran) and Rifampicin from Alhavi Pharmaceutical Company (Iran). Using standard methods, the antibiotics were prepared in appropriate concentrations and divided into 1.5 ml vials and stored at -20 °C. MS culture medium[15] was used for plant tissue cultivation. *Nicotiana tabacum* (Var. Xanti) seeds were obtained from Seed and Plant Improvement Institute (Karaj, Iran) and kept at 4 °C until us.

Designing of the Chimeric Gene

To stimulate a potent immune response, two immunogens of enterotoxigenic *E. coli*, including CfaB and Eae were selected to be incorporated into the chimeric antigen. Indeed, two *E. coli* -derived adjuvants, the binding domain of shiga-like toxin (from enterohaemorrhagic *E. coli*) and the binding domain of the heat-labile toxin (from enterotoxigenic *E. coli*) were added at each terminus (C-terminus and N-terminus, respectively) of the chimeric antigen. Accordingly, the chimeric antigen was abbreviated to SECL.

The final arrangement of the chimeric antigen's components has been presented in the Figure 1. EAAAK linker was employed to separate the different antigens. The final structure had 659 amino acid residues with the molecular weight of about 65 kDa. As it was mentioned before, the characteristics of the chimeric protein has been explained previously.[16]

The final protein was reverse-translated and codon-optimized according to plants codon preference and synthesized in pBI121 vector by Biomatic Company (Canada).

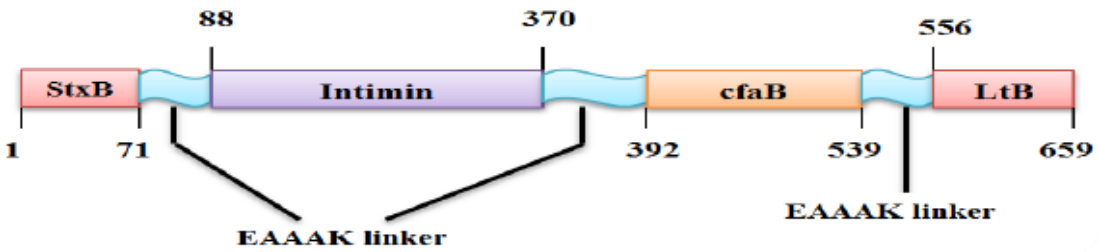

Figure 1. The Arrangement of Immunogens in the Chimeric Antigen. EAAAK linker was used for efficient separation of the immunogens and the final antigen has 659 amino acid residues.

Transferring the Recombinant Plasmid into E. coli Cells

pBI121 recombinant plasmids containing *secl* recombinant gene were transferred into *E. coli* cells to have sufficient amount of the recombinant plasmid. Cells were competent using chemical method and transformation were performed via heat-shock method.[17] To confirm the transformation of bacterial cells, colony PCR was performed using specific primers and the results were evaluated using agarose gel electrophoresis (1.5%). The sequence of primers was as follows: F primer: ACAATGGCAGACTGTGCAAAG; R primer: TCAAAGTTCGTCCTTGTGATGATG.

Transformation of Agrobacterium Bacteria

The recombinant vector was extracted by alkaline lysis method[18] and transferred into *Agrobacterium tumefaciens* strain LBA4404 and *Agrobacterium rhizogenes* (ATCC 15834) by freeze and thaw method using 29 mM calcium chloride solution and liquid nitrogen as described by Sambrook et al.[17] Since the bacterial cells have been cultured on medium containing two antibiotics, kanamycin and rifampicin, the transformation process can be largely assured. However, for further confirmation, we performed colony PCR using specific primers (F primer: GTTGAGAA GAACATTACCGTTACAGC; R primer: GAGTCATAAC AAGAGAAACAACACCGG).

Transformation of Tobacco Plants for the Induction of Hairy Roots

For the induction of hairy roots in the plants, recombinant *Agrobacterium rhizogenes* (ATCC 15834) was cultivated in LB medium containing 50 mg/L of kanamycin and 30 mg/L rifampicin for an overnight. The medium was incubated at

28 °C with shaking (150 rpm). Bacteria were harvested under sterile conditions by centrifugation at 860 g for 10 min in 4 °C. The supernatant was discarded and 5-10 ml of 5% MS solution containing 5% glucose and 100 µM sterile acetosyringe to optical absorption at 600 nm reached to 0.5 and again it was incubated for 2 hours at 28 °C and shaking at 180 rpm. The bacterial suspensions of recombinant bacteria were separately transferred to a sterile petri dish under sterile conditions. For tobacco plant transformation, a 2 x 2 cm crosswise from sterile and young leaves of a one-month-old plant was incubated along with the recombinant bacteria so that the upper surface of the cut leaves was exposed on the medium. The plates were placed in an aluminum foil at room temperature and in a dark place. After 48 hours, the leaves were washed in sterile distilled water containing 300 mg/L cefotaxime and dried on a sterile filter paper. Then, tobacco samples were transferred to a medium containing 50 mg/L kanamycin and petri dishes were exposed to moderately bright light (1000 lux) for 16 hours and darkness for 8 h at 25 °C till the hairy roots of the tobacco emerge from the edges. Following the emergence of the roots, they were classified by transferring the single root to the newly selective medium. Once every 7 days, one piece of root of each category was transferred to a newly selective medium with a higher concentration of kanamycin (100 mg/L). Then, the roots were transferred to liquid MS medium in Erlenmeyer for further growth. Erlenmeyer was incubated at 25 °C with shaking of 149 rpm for two weeks, when the grown roots were harvested and kept at -70 °C for further analysis.

Confirmation of the Hairy Roots' Transformation

For molecular analysis of transgenic hairy roots, the presence of the *secl* gene was investigated in the roots. For this aim, firstly, the roots' genomic DNA was extracted by CTAB (Cetyl trimethyl ammonium bromide) method, as described by[19] and evaluated by agarose gel electrophoresis as well as spectrophotometry at 260 and 280 nm. Then PCR was performed by FCF and RLT-specific primers which amplify a region within *cfab* and *ltb* genes in the 3'OH terminus of the chimeric gene. The sequence of primers was as follows: F primer: ACAATGGCAGACTGTGCAAAG; R primer: TCAAAGTTCGTCCTTGTGATGATG.

Extraction of the Roots' Total Protein

To extract the total protein of the roots, 150 mg of roots were frozen in liquid nitrogen and then ground. Total protein was extracted manually according to Wink et al.[20]

Determination of SECL Chimeric Protein Expression by Semi-quantitative ELISA

To measure the amount of the expressed recombinant SECL protein in hairy roots, semi-quantitative indirect ELISA method was exploited. The method was as described by Crowther et al.[21] For this purpose, 25 ng of total protein purified from the root was coated in each well. A homemade antibody against SECL protein, that was prepared previously in our laboratory in mice, was used as the primary antibody. Anti-mouse IgG antibody, HRP conjugate was used as the secondary antibody and at last the results were read by an ELISA reader.

Then, the amounts of recombinant SECL protein in the hairy roots were calculated using direct and indirect methods by a standard diagram drawn using different amounts of recombinant SECL protein.

Results

Transformation of E. coli Cells

pBI121 recombinant plasmid carrying the *sicl* gene was transferred to the competent *E. coli* cells by heat-shock method and the screening was done by culturing the transformed cells on LB agar medium. The transformation of *E. coli* cells was confirmed by colony PCR using specific primers as follows: F primer: ACAATGGCAGACTGTGC AAAG; R primer: TCAAAGTTCGTCCTTGTGATGATG. PCR products will have an 825 bp length. As can be seen in Figure 2, out of four evaluated colonies, three had gained the recombinant plasmid and were transformed.

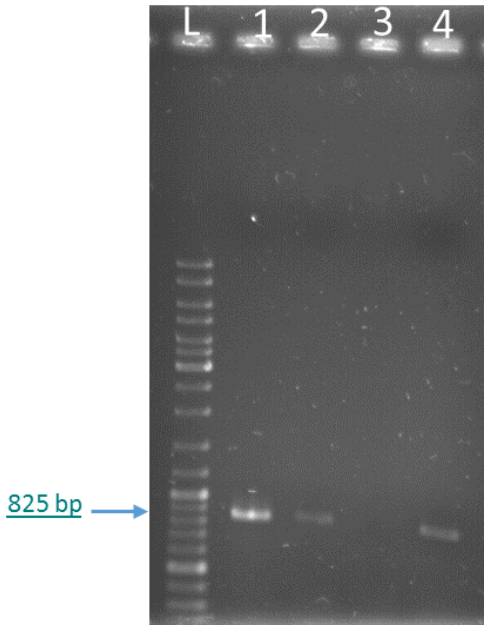

Figure 2. Confirmation of Bacterial Transformation Using Colony PCR. Lane 1: clone 1; Lane 2: clone 2; Lane 3: negative control; Lane 4: clone 3; L: 100 bp ladder.

Transformation of Agrobacterium rhizogenes

Recombinant plasmids were extracted from transformed *E. coli* cells and used for the transformation of *Agrobacterium rhizogenes* bacteria. The transformation of *Agrobacterium rhizogenes* was confirmed by colony PCR using specific

primers (F primer: GTTGAGAAGAACATTACCGTTACA GC; R primer: GAGTCATAACAAGAGAAACAACACCG G) by them a 433 bp fragment is amplified. As can be seen in Figure 3, out of three evaluated colonies, two of them had gained the recombinant plasmid.

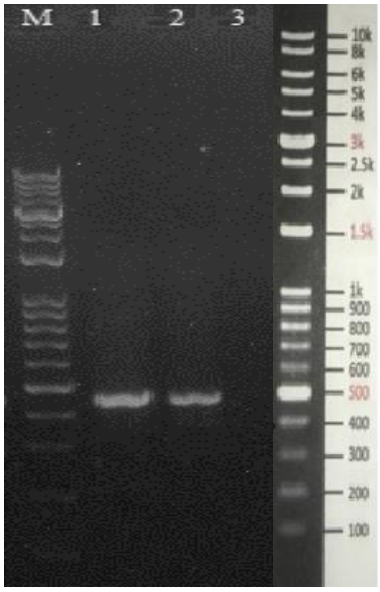

Figure 3. Confirmation of Bacterial Transformation Using Colony PCR. Lane 1: clone 1; Lane 2: clone 2; Lane 3: clone 3; M: 1 kb DNA ladder.

Transformation of Tobacco Plants with Recombinant Agrobacterium rhizogenes for the Induction of Hairy Roots and the Confirmation of Transformation

Following the transformation of tobacco plant and induction of hairy roots, the genomes of the hairy roots were extracted by Edward method.[22] For the confirmation of the presence of genes responsible for hairy root phenotype in the plant genome, PCR reaction was performed with specific primers for *Agrobacterium rolB* gene, which is one of the genes involved in the development of this phenotype and is simultaneously inserted into the plant genome with T-DNA. As figure 4 shows, a 433 bp fragment has been amplified which indicates the presence of this gene in the plant genome.

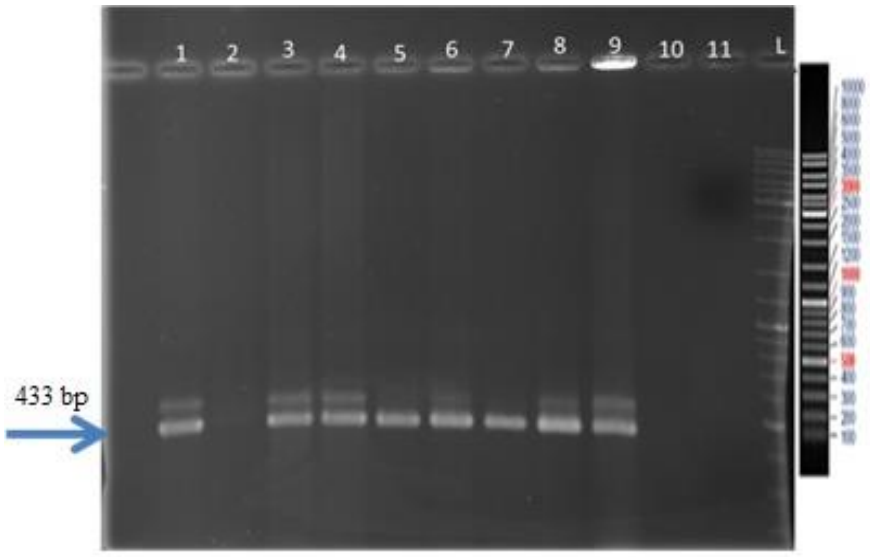

Figure 4. Conformation of the Presence of Genes Involved in Hairy Root Phenotype Using Specific Primers of *rolB* Gene. Lanes 1-4: PCR reaction product from hairy root genomic DNA by indirect method; Lanes 5-8: PCR product from hairy root genomic DNA by direct method; Lane 9: Positive control (PCR colony on recombinant *Agrobacterium*); Lanes 10 and 11: Negative controls (Products of PCR reaction on genomic DNA of non-transgenic tobacco roots); L: 100 bp DNA molecular weight marker (SM0331, Fermentas).

To illustrate the non-contamination of transgenic hairy roots with *Agrobacterium rhizogenesis*, PCR reaction with specific primers of *virG* gene was performed (Figure 5). The results showed that there is no contamination with this bacterium. In positive control, a 400 bp fragment has been amplified that shows the correctness of the PCR reaction.

Confirmation of the Presence of the Chimeric Gene in Hairy Roots

To confirm the presence of *secl* chimeric gene in hairy roots, PCR was performed using specific primers. Extracted genomic DNA was used as a template in the PCR reaction. Figure 6 shows the results of this test. As it was anticipated, a 825 bp fragment has been amplified in the majority of the samples which proves the presence of *secl* gene in the plants.

Evaluation of SECL Recombinant Protein Production in Plants and Transgenic Hairy Roots

To determine the production of recombinant SECL protein in transgenic hairy roots, after the extraction of the total protein from these roots and determination of the protein amount by Bradford method, ELISA method was used to measure the SECL protein produced in the plant. For this experiment, an antibody produced in mice immunized with recombinant SECL

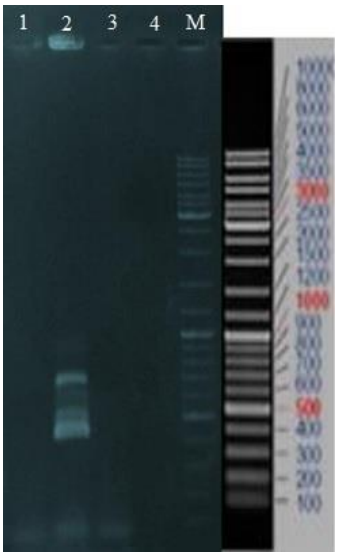

Figure 5. Confirmation of non-Contamination of Plants with *Agrobacterium* through PCR Reaction with *virG* Gene Primers on Genomic DNA Extracted from the Plants. Lanes 1 and 3: PCR product from genomic DNA of transgenic plants; Lane 2: positive control (PCR reaction product on Agrobacterium colony); Lane 4: nothing is loaded; M: 100 bp DNA ladder (Fermentas, SM0331).

protein (of prokaryotic origin prepared in our laboratory) was used as the primary antibody. After reading the absorbance of each sample at 492 nm, the amounts of recombinant SECL protein in the hairy roots were calculated from a standard diagram drawn using different amounts of recombinant SECL protein. Then the amount of SECL protein was calculated. The results show that the recombinant expression of the protein in the direct and indirect methods was 0.00 to 0.0012% of total soluble proteins (TSP), respectively (Figure 7).

Discussion

In the present study, a chimeric antigen, as a vaccine candidate against two diarrheagenic *Escherichia coli* species, enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) was cloned and expressed in hairy roots of *N. tabaccum* plants. In this four-components antigen, one antigen was related to EHEC (the binding region of shiga-like toxin (StxB)) and three antigens were originated from ETEC (the binding region of the heat-labile toxin (LTB), binding factors, including intimin (an attachment factor), and CFA (a colonization factor)). The details of the structure and

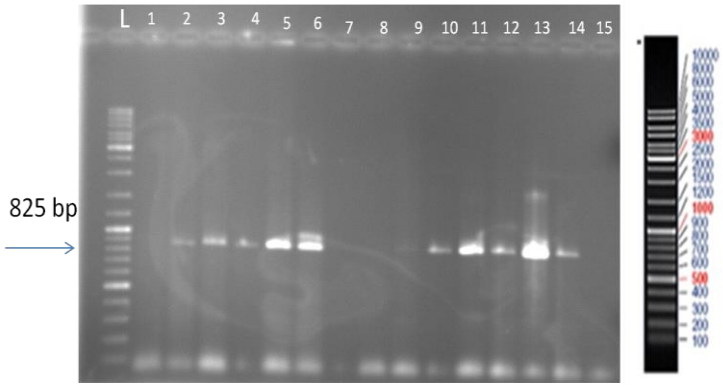

Figure 6. Investigation of the Presence of pBI 121 + *sec*/ Gene Construct in the Hairy Roots Obtained from Indirect and Direct Methods by PCR. L: 100 bp DNA ladder (SM0331, Fermentas); Lanes 1 to 5: PCR products from genomic DNA of hairy roots obtained by indirect method; Lanes 6 to 12: PCR product from genomic DNA of the hairy roots obtained by direct method; Lanes 13 and 14 PCR product from genomic DNA from *Agrobacterium rhizogenesis* (positive control); Lane 15: PCR product from genomic DNA from non-transgenic tobacco root (negative control).

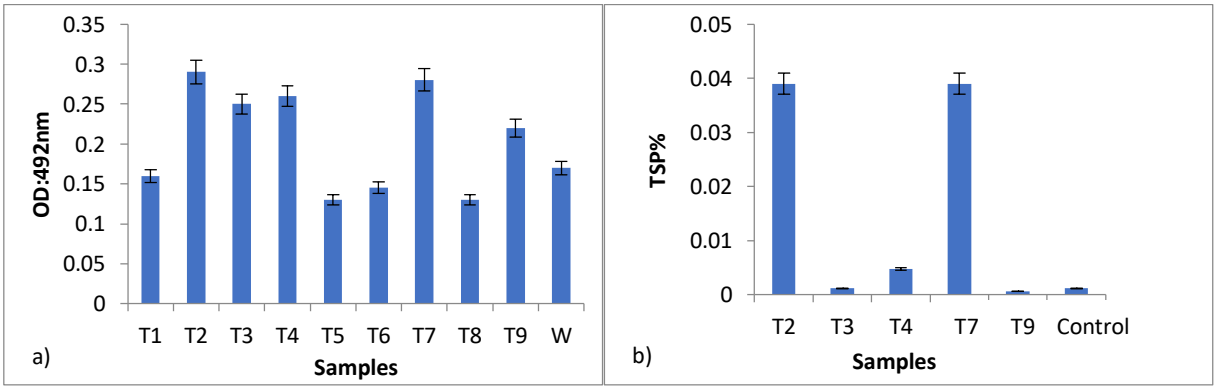

Figure 7. Investigation of the Protein Expression in Hairy Roots in Direct and Indirect Methods. **a)** According to the calculations based on the OD of the samples, the samples that were less than a defined limit (T1, T5, T6, T8) were removed. **b)** For the remaining samples, the amount of SECL protein produced was calculated based on the percentage of TSP.

information of this chimeric antigen has been described previously.[16]

Hairy roots are one of the tissues used to produce recombinant proteins.[23,24] Agrobacteria are good vehicles to transfer the desired genes into the host plant nucleus. *Agrobacterium tumefaciens* is able to insert the desired genes into the plant's nuclear genome and, by simultaneous production of auxin and cytokinin hormones, produce developed plant tissues without differentiation (gall). But in *Agrobacterium rhizogenesis*, the production of the cytokinin hormone is practically disrupted and the genes transferred into the nucleus of the plant cell can only produce the auxin hormone. In this case, only the root is created in the infected tissues. *Agrobacterium rhizogenesis* transfers part of its plasmid into the plant's genome. This part contains genes involved in the synthesis of plant hormones, which induce the production of hairy roots.[25]

The expression of bacterial antigens as immunogens in different plant systems and different parts, such as edible leaves of cereals and legumes, plant storage parts, such as potatoes, fruits and roots, has received much attention. Historically, in 1997, peroxidase was expressed by the hairy roots of *Brassica napus*.[26] The expression of important antigens in immunogenicity against viral pathogens was considered by the researchers. In the first successful reports, Kumar et al. produced significant production for hepatitis B virus surface protein (HBsAg) of transgenic plants from direct gene transfer with *A. tumefacience* or regenerated plants from hairy roots (between 13.4 to 19.1 ng/g fresh plant).[27] By changing the host, the expression of HBsAg in potato hairy roots reached to 97.1 ng/g of fresh weight. However, in transgenic plants resulting from *A. tumefacience* gene transfer or regenerated plants from hairy roots, the expression level of HBsAg was significantly reduced (19.1 and 13.4 ng/g of fresh weight, respectively).[27] A significant increase in human acetylcholinesterase up to 3.3% TSP has been reported for hairy roots in *Nicotiana benthamiana*, obtained by *A. rhizogenes* gene transfer. However, acetylcholinesterase expression in the parent plant was three times less than the hairy root.[28]

In 2012, the thaumatin protein was expressed in the hairy roots of the tobacco plant. The maximum expression was reported 1.4% and 0.13% of TSP in the structure containing signal peptide and without signal peptide, respectively.[20] In 2010, Topal expressed the carboxyl terminus of intimin in tomato seeds and compared its immunogenicity with the chimeric protein expressing the carboxyl terminus of intimin and StxB. Based on the results of this study, mice treated with this chimeric protein showed a better immune response than treatment with separate administration of StxB protein and the carboxyl terminus of intimin.[29] In all these cases, it was found that plants are able to produce immunogenic substances and can be used as a useful and effective host in the production of vaccine raw materials.

In this study, the chimeric *secl* gene was synthesized and cloned into an expression vector and transferred into the plant in two ways: direct method, using recombinant *A. rhizogenes* containing *secl* gene controlled by 35s CaMV promoter; and indirect method, in which at first, transfection was performed with recombinant *A. tumefaciens* containing the *secl* and then rooting was induced by non-recombinant *A. rhizogenes*. In the direct method, it is possible that the hairy roots grown on the selected medium lack our desired gene. For this reason, we use the indirect method that rooting is performed by non-recombinant *Agrobacterium*. After examining the generated hairy roots, the results showed that in the indirect method, the microsamples began to generate root much faster than the direct method. The reason for this difference may be the resistance of the microsamples to kanamycin. It has also been observed that larger leaves are more susceptible to rooting than smaller ones, perhaps due to the release of phenolic compounds as well as the release of sugar compounds, such as glucose and pH-lowering substances from vacuoles, which all stimulate the improved acceptance of the desired DNA by plants.

In this study, hairy root growth was studied sequentially in selective media with gradual increase in the concentration of kanamycin (25, 50, 75, and 100 µg/µl). The results showed that under these conditions (gradual increase in kanamycin concentration), more root production is observed in plants; while the sudden use of high kanamycin concentration (100 µg/µl) reduces the root density. Sudden pressure from the antibiotic appears to cause rapid cell death and the release of acidic substances and intracellular contents. This can also endanger the survival of suitable and transgenic cells and practically reduce the rate. However, in the gradual increase of the selective factor, less cell death occurs in the early stages and there will be more favorable conditions for the expression of the antibiotic resistance gene in the transformed cells. In this case, the remaining cells resist at higher concentrations of the selective agent and proliferate, resulting in more hairy roots. These results are consistent with previous reports and the group experiences.[30]

Numerous previous studies have shown that the heterologous protein expression in hairy roots can be up to seven times as much as the leaves. Hairy roots are metabolically so active and mRNA levels in these structure are too high.[27,31] According to studies conducted in 2015, the expression of recombinant ranalexin protein (a new antimicrobial peptide derived from rabbit skin and structurally is similar to polymyxin antibiotic) in transgenic plants has occurred in hairy roots in two different conditions. In the presence of signal peptide, the expression level reached to 3.3% of TSP; while in the absence of signal peptide, in the best situations the protein expression was 0.83% of TSP after 20 days (about 4 times lower).[32] In this study, the evaluation of the

expression level of the SECL protein was determined by ELISA. In the indirect method, the maximum protein expression was calculated to be 0.039% of TSP and in the direct method it was 0.0012% of TSP. The high expression of protein in the indirect method is also consistent with the previously reported results.[30] The amount of SECL protein expressed in the hairy roots in indirect method was approximately 8 times that of the direct method. Although this does not seem to be a good deal compared to other smaller proteins, the ability to express proteins with a high molecular weight (about 70 kDa) is a significant finding. Obviously, placing the signal sequence to secrete the product into the culture medium of hairy roots can increase the protein production.[32] But in this case, the main goal is to introduce the roots as a source of antigen (for immunization), do it is better that the protein remains in the roots to avoid destruction. However, it has been shown that multiple sign sequences must be used to transfer proteins to the extracellular space and culture medium. These peptide sequences include the patatin guide peptide from potato storage protein for the secretion of the Mgfp5 reporter gene into tobacco hairy root culture medium[33] and the peptide guide peptide methyl esterase from Arabidopsis for G He pointed to the capillary roots of tobacco and rapeseed[34] to introduce the best synopsis with the best yield for this protein (SECL). The probable reason is that the structure is chimeric. In fact, being a chimerical system is unfamiliar to the host for which there is no metabolic definition.

Conclusion

In conclusion, in the present study, the efficiency of hairy roots of tobacco plant as a proper expression system for the recombinant production of a chimeric recombinant SECL protein was shown. This system can be exploited for heterologous expression of other proteins. Indeed, the SECL protein can be used for mice's immunization against enterotoxigenic and enterohemmorhagic *E. coli* strains.

Authors' Contributions

study conception and design by AHS and JA; do experiments and data collection by AA and MJ; analysis and interpretation of results by AHS, AA, and JA; draft manuscript preparation by AA and FF. All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

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