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Original Article

The Immunogenicity of a YncE Antigen from *E. coli Enterotoxigenic* (ETEC) by Edible Delivery of Transgenic Hairy Roots Tobacco

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Abstract

Introduction: One of the most causes of diarrhea disease is *Escherichia coli enterotoxigenic* (ETEC). The first stage of the disease is the binding of ETEC to small intestinal epithelial cells by colonization factors. ETEC then produces Heat-labile enterotoxin (LT) and heat-stable (ST) enterotoxins. The *yncE* gene potentially encodes a protein with sequence similarity to a pyrroloquinoline quinone containing periplasmic oxidase. The *YncE* gene is conserved in various strains of *E. coli*. The protein encoded by the *YncE* gene is present in the membrane structure of the ETEC. The *YncE* gene can be considered as a novel protective vaccine candidate. The aim of the present study was to investigate herbal vaccines as a solution to health problems, especially in developing countries.

Materials and Methods: In the present study, the expression of the YncE protein in tobacco hairy roots and its immunogenicity in mice were investigated. The *YncE* gene was cloned downstream of CaMV-35S promoter in the binary expression vector, pBI121-YncE, by using different strains of the *Agrobacterium rhizogenes* (A4, MSU, 15834) and LBA4404. Three groups of mice including edible, edible-injection, and control were examined. ELISA method was used to determination of IgG and IgA in Feces and Serum.

Results: The amount of the YncE protein was estimated approximately 0.9% of the Total Soluble Protein (TSP) in the transgenic hairy roots. The results indicate that the recombinant YncE protein produced in the transgenic hairy roots tobacco was able to stimulate the immune response in BALB/c mice. Also, it can be stated that recombinant YncE protein is an effective immunogen. The results implied the potential of transgenic tobacco hairy roots–based expression for oral-injection and oral delivery of YncE protein. The antibody titer showed that the immune system was well stimulated.

Conclusions: The plant-based vaccine recombinant YncE protein could both humoral and mucosal the immune response, effectively. Therefore, the YncE antigen was able to stimulate the immune system of mice and produce antibodies. Actually, it appears to be a suitable vaccine candidate for ETEC-induced diarrhea.

Keywords: Diarrheal Disease, *E. coli Enterotoxigenic*, YncE Protein, Transgenic Tobacco, Hairy Roots, Oral Immunization

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Introduction

Diarrhea is an important disease in developing countries. It is worth mentioning that over 1.6 million people died from diarrheal diseases in the world, in 2017. Children under five years old are considered to be one-third of all who died from diarrheal diseases.[1] This disease is endemic in some countries such as Latin America, Sub-Saharan Africa and South Asia.[2] Many factors cause this disease such as viruses (types of viruses that cause diarrhea include: cytomegalovirus and viral hepatitis), some medications (such as antibiotics, anti-cancer drugs), lactose intolerance and bacteria. One of the most important pathogens causing bacterial diarrhea is *E. coli enterotoxigenic* (ETEC). Children die every year as a result of this disease and a number of them are malnourished.[3]

One of the most important components in diarrhea disease is ETEC. Colonization Factors (CFs) and enterotoxins are two main virulence factors of Enterotoxigenic *Escherichia coli*. The mechanism of infection is that ETEC is at first attached to the small intestinal epithelial cells by surface proteins called fimbrial colonization. Different types of CFs such as CFA/II, CS1, CS2, and CFA/IV have been identified and approximately 70% of cases are caused by ETEC diarrhea.[4] After the CFs attach to cells, ETEC produces two types of enterotoxins, one of which is heat-labile (LT) and the other heat-stable (ST). The encoding genes of these two

enterotoxins are located on the plasmid. These two enterotoxins can both separately and simultaneously cause diarrheal disease.[5]

Due to the fact that the resistance of bacteria to antibiotics (macrolides and penicillin) is increasing, one of the key factors in controlling diarrhea is vaccination.[6] One of the applied technologies in this field are plant-based vaccines. Edible vaccines are extremely effective for bacteria and actually target the gastrointestinal tract as they stimulate the mucosal immune system.[7]

Transgenic plants are a good source for the production of oral vaccines because the recombinant proteins produced in the plant are used orally and can stimulate the mucosal immune system well.[8,9] Oral vaccines also have many benefits such as lacking common pathogens between humans and animals, no need for a cold chain, syringe and injection and the recombinant protein produced in transgenic plants has the correct function because of performing post-translational modification such as glycosylation.[10] There are several methods for transferring foreign genes into plants. One of the most important methods is *Agrobacterium rhizogenes* because the hair roots produced in the plant have many advantages including growing rapidly, genetically stable, needing a hormone-free culture medium and increasing the production of recombinant proteins and secondary metabolites.

The *YncE* gene is conserved in a variety of bacterial strains of *Escherichia coli*. Its physiological function is not yet known.[11] The protein encoded by the *YncE* gene is present in the membrane structure of the *E. coli* and a decrease in the expression of this protein leads a disorder in the structure of the bacterial membrane.[12] The structure of YncE protein is defined by a typical seven-bladed β-propeller.[13] The expression of this protein is affected by the limitations of Fe^{2+}.[14] Previous research has shown that the immune system is highly stimulated after the YncE antigen vaccine is injected into a mouse and the amount of IgG in the blood of mice was greatly increased and they were protected against the infection.[14] Therefore, if YncE protein is produced in a suitable host such as a transgenic plant, it can be considered as an edible vaccine candidate.

The current study was based on producing YncE protein in the transgenic tobacco plant by the *A. rhizogenesis* method by different strains such as A4, MSU, and AR15834. We also intended to confirm the transferring of the rol B gene into the transgenic hair roots by PCR and specific primers. In addition, we evaluated the expression levels of the *YncE* gene and also the production of recombinant YncE protein in the tobacco plant hair roots and evaluated its immunogenicity in a mouse model.

Materials and Methods

Specifically Designing Primers YncE gene and Cloning into Plant Expression Vector

ETEC is a source of *YncE* gene. *YncE* gene was subcloned in XbaI/SacI sites of the pBI121 vector (Novagen, USA). In the vector, the *YncE* gene replaced the *β-glucuronidase* gene. The *YncE* gene was amplified using specific primers for cloning into the pBI121 vector. The forward primer contains an ATG codon, Xba1 site, and His-tag sequence as well as to enhance the efficiency of protein production, the Kozak sequence. The reverse primer contains His-tag, the Sac1 site, and KDEL sequences which fused to the 3′ end of the *YncE* gene, a KDEL endoplasmic reticulum retention signal sequence, and a stop codon. In pBI121, the *YncE* gene was cloned downstream of the Cauliflower Mosaic Virus35S (CaMV35) promoter. The bioinformatics analysis was performed as described previously.[15] PCR product and pBI121 plasmid containing *gus* gene were double digested with XbaI and SacI by restriction enzyme buffer (Thermo Scientific). *YncE* gene sub cloned into XbaI/SacI sites of pBI121 expression vectors (Clontech Co.). The confirmation of the accurate clones was done by PCR and restriction enzymes analysis. Finally, plasmid pBI121-YncE was transformed into *A. tumefaciens* (LBA4404) and *A. rhizogenes* (MSU, A4 and 15834) by using the freeze–thaw method.[16]

Generation of Transgenic Tobacco Hairy Root Lines

Seeds of *N. tabacum* cv. Samson (National Research Institute of Genetic Engineering, Tehran, Iran) were sterilized by 2% NaCl (w/v) for 10 min and washed with sterile water three times. Then, ½ Murashige and Skoog (MS) culture medium was prepared and the seeds were transferred to the plates.[17] The seeds germinated about seven days later and were transferred to a big jar. After three weeks, the leaf segments were cut and immersed in the *A. rhizogenes* suspension for 5 min. After drying on filter paper, they were co-cultured with the *A. rhizogenes* harboring pBI121-YncE for two days in darkness.[18] The explants were transferred to the fresh and selective medium supplemented with 50 mg/L of kanamycin and 300 mg/L of cefotaxime in a phytotron under a 16/8-h light/dark photoperiod. They were kept in the greenhouse until the hair roots were produced.

DNA Extraction and PCR Analysis of Transgenic Tobacco Hairy Root Lines

To confirm the presence of the target gene and to validate the transformation, PCR analysis was performed. For this purpose, the extraction of the total genomic DNA from hairy roots (1 g) of different transgenic lines and non-transgenic hairy root (negative control) were done by the CTAB (cetyltrimethylammonium bromide) method. Briefly, for each 100 mg tissue, 500 µl CTAB extraction buffer was used at 60 °C for 30 min, following centrifuge for 5 min at up to 14000 × g and then the supernatant transferred to a new tube. In the following, 5 µl RNase added to the supernatant and incubated at 37 °C for 20 min and after that, the content of the tube were centrifuged for 1 min at 14000 × g. The

upper aqueous phase was transferred to a new tube and added 0.7 volume cold isopropanol and incubated at -20 °C for 15 min. The pellet was washed with 70% ethanol and air dried in room temperature. Then, 20 µl of TE buffer was added to dissolve the DNA.[19] The forward primer (YncE+2F) 5-GCGCTCTAGAAAAACAATGGCAGAAGAAATGCTG CGT-3 and the reverse primer (YncER) 5-ATTAGAGCTCT TAAAGCTCATCTTTGTGGTGGTGGTGGT-3 primers were used for PCR analysis. PCR amplification was set up: 3 min at 95 °C for initial denaturation; 40 cycles of 20 sec at 94 °C for melting, 20 sec at 59 °C for annealing, and 30 sec at 72 °C for extension; and then 5 min at 72 °C for the final extension. The PCR analysis for the *rolB* gene with forward 5-GCTCTTGCAGTGCTAGATTT-3 and reverse 5-GAAG GTGCAAGCTACCTCTC-3 primers was used to confirm the presence of hairy roots. In addition, the PCR with virG forward 5-AGTTCAATCGTGTACTTTCCT-3 and reverse 5-CTGATATTCAGTGTCCAGTCT-3 primers were used to confirm the transformation and *Agrobacterium* contamination.[20] The amplified DNA fragment was run on 1% agarose gel electrophoresis and visualized under UV light.

Extraction of Protein

Using liquid nitrogen, all soluble proteins were extracted from three of the best hairy root lines. Hairy roots were ground in the mortar using liquid nitrogen and transferred to the micro tube. Extraction buffer (200 mM Tris–HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 15 mM 2-mercaptoethanol, 1 Mm PMSF, 0.05% Tween-20) was added to the tube. Vortex and centrifugation (13000 × g for 10 min at 4° C) were performed.[21] The amount of the Total Solution Protein (TSP) in the supernatant was then determined using the standard Bradford method.[22] The standard ELISA method was also used. Micro well plates were coated with 10 µg TSP in carbonate buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) and incubated overnight at 4 °C. The maxisorp plate was blocked using 3% (w/v) non-fat skim milk in PBS buffer containing 0.05% Tween-20 (PBST) and washed by PBST 3 times. The microplate was incubated with an HRP-conjugated goat (Sigma Co. Germany) for 90 min at 37 °C. O-phenylenediamine (OPD) (Sigma Co. Germany) solution plus H_2O_2 was used for color development and 100 µl of Na_2HPO_4 was added to each well to stop the reaction. The optical density titers were measured at 492 nm.

Western Blot Analysis of Transgenic Plants

Detection of YncE protein in transgenic plants was carried out by immunoblotting methods. Western blot analysis was performed for three best lines of transgenic hairy roots. The bands on the SDS-PAGE were transferred to the Polyvinylidene Difluoride (PVDF) membrane (Roche Co. Germany) using transfer buffer (50 mM Tris base, 40 mM glycine, pH 8.3). The PVDF was blocked using 5% non-fat milk powder in TBS with 0.05% Tween-20 (TBST) at 37 °C for 2 h and then washed twice for 5 min in TBST. For detecting the YncE protein, the membrane was incubated with a monoclonal anti-his HRP conjugate antibody (1:2000 dilution) for 1.5 h at 37 °C.

Mice Immunization

Three groups of female BALB/c mice (purchased from Razi Vaccine and Serum Research Institute, Karaj, Iran) were examined. There were five mice in each group. 25 µg recombinant YncE was prescribed in each group including edible and edible-injection for 4 times with 7-day intervals. Blood sampling was performed in all groups one week after the third and fourth stages.[23]

ELISA for Determine IgG and IgA in Feces and Serum

Blood samples were transferred into the microtubule and heated to 37° C for one hour and then centrifuged at 10000 rpm for 5 min. The determination of IgA levels was performed by collecting the fecal and then mixing with 500 µl PBS containing protease inhibitors and storing overnight at 4 °C and then centrifuged for 5 min and 1000 rpm.[23] Serum antibody titer against YncE was determined using conjugated IgG (1:5000 dilution) and IgA (1:8000 dilution) (Sigma, Germany) as the secondary antibody.

Results

Design and Cloning of the YncE Gene

The translational enhancer (Kozak sequence) and his-tag sequence were inserted in the forward primer before the start codon. In the reverse primer his-tag sequence and endoplasmic retention signal sequence (KDEL) were introduced. The *YncE* gene (972 bp) was subcloned into the pBI121bin 1ary vectors for expression in plant cells under the control of the CaMV35S promoter.

Molecular Analysis of Transgenic Plants

The pBI121-YncE vector was used for the stable transformation of tobacco plants by *A. rhizogenes* (MSU, A4, and 15834) and *A. tumefaciens* (LBA4404). The hairy roots appeared in the selective medium containing 50 µg/ml kanamycin (Figure 1a). After extraction of genomic DNA from nine lines, PCR analysis was performed using specific primers, which the most obvious bands belong to A4 (Figure 1b). PCR confirmed the rolB integration in a transgenic plant, and a fragment of the same size (386 bp) was amplified from of the four hairy root lines (Figure 1c). A fragment with a similar size was also amplified from the plasmid DNA of *A. rhizogenes* MSU (positive control). The *virG* gene was not amplified from any of the four transgenic hairy root lines, confirming the true transformation of hairy roots without *A. rhizogenes* contamination (Figure 1d).

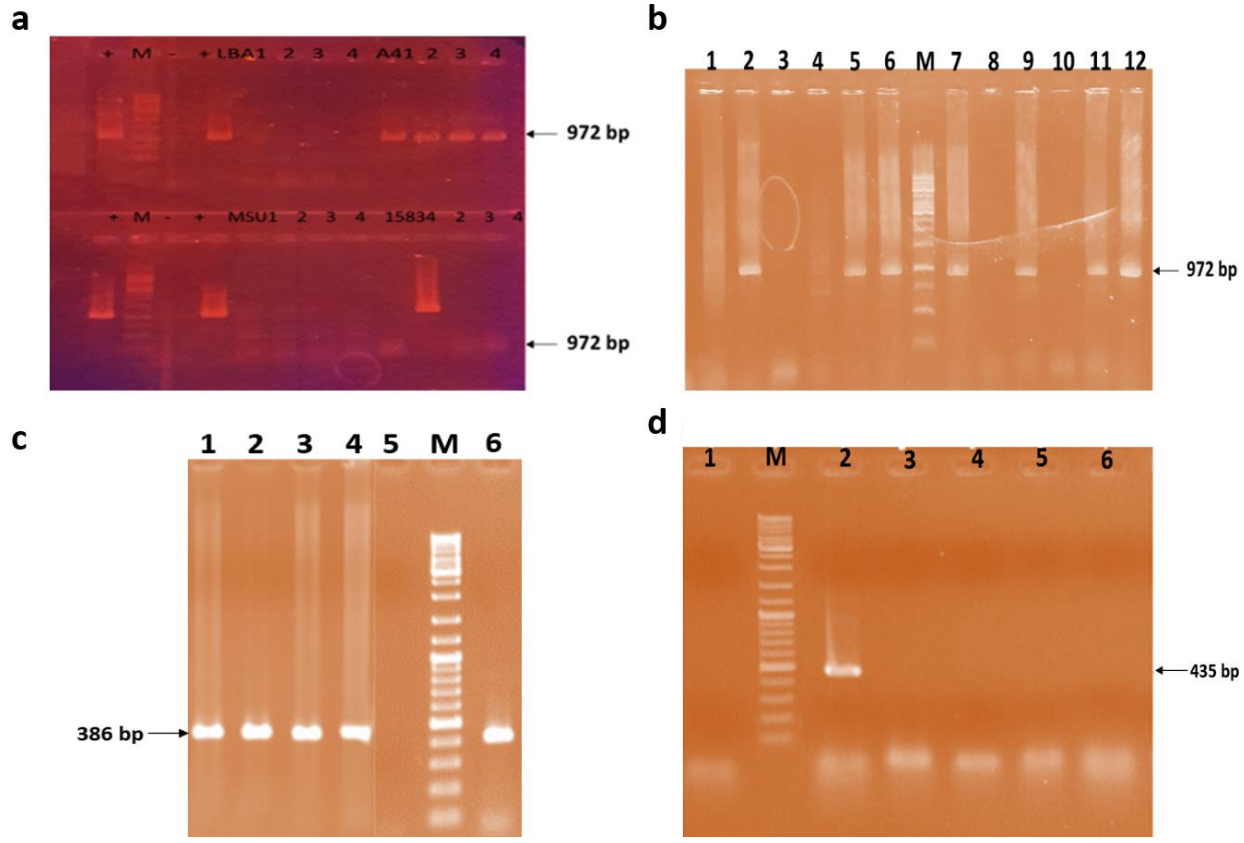


Figure 1. Confirmation of Transgenic Root by PCR Methods. **a)** Confirmation of present the recombinant pBI121vector (containing gene of interest) in *agrobacterium* by colony-PCR; Column +(1): positive control reaction, pET28a- YncE; Column +(2): positive control reaction, pBI121-YncE; Column M: DNA size marker; Column -: negative control (non-transgenic *Agrobacterium*), the rest of the columns belong to different *Agrobacterium* strains (A4, 15834, MSU A, and LBA4404. The most obvious bands belong to A4. **b)** PCR test on genomic DNA extracted from transgenic plants using specific primers *YncE* gene; Column1: PCR products from genomic DNA extracted from non-transgenic (negative control); Columns 2: positive control reaction (pBI121-YncE); Columns 5, 6, 7, 9, 11, and 12: containing target gene; Column M: Molecular weight markers mix (Fermentas). **c)** The PCR technique for the *rolB* gene whit specific primer was used to confirm the presence of hairy roots. Columns 1, 2, 3, and 4: containing *RolB* gene. Its size is 386 bp. Column M: Molecular weight markers mix (Fermentas); Column 6: positive control (also amplified from the plasmid DNA of *A. rhizogenes* AR15834); Column 5: negative control (nontransformed root). **d)** To confirm the correct transformation of hairy roots without bacterial contamination, PCR amplification included the *virG* gene. Line 1 refers to the non transformed root. Lane M refers to DNA ladder. Lane 2: negative control (non transformed root). Lanes 3-6 show the absence of the *virG* gene in the 4 transgenic hairy root lines.

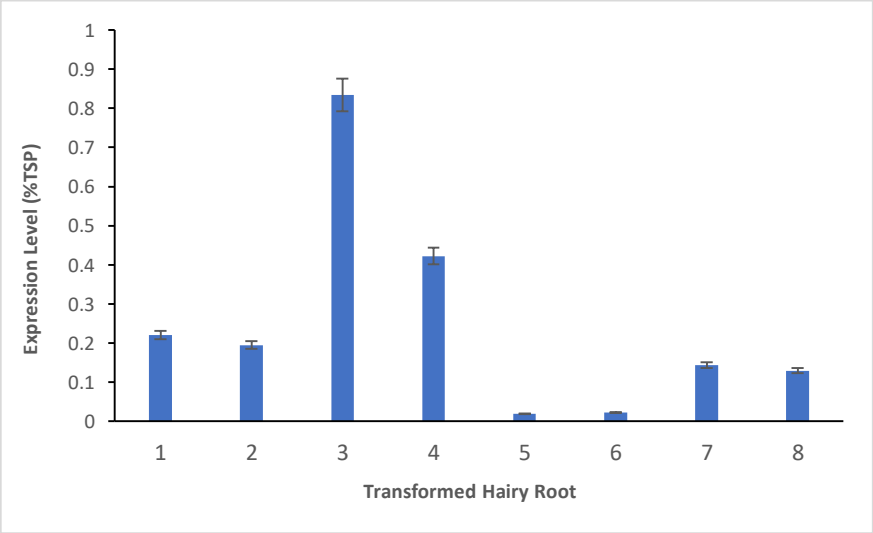


Figure 2. Measurement of YncE Protein in Transgenic Tobacco Hairy Roots by Quantitative ELISA (1-5: different transgenic lines). The level of expression was estimated as approximately 0.9% of TSP in tobacco hairy roots. The absorbance value is the optical density values at 492 nm obtained from three independent assays.

Expression of YncE Protein in Tobacco Hairy Roots

The analysis of protein extraction showed that there are two transgenic hairy roots (Lines 3 and 4). These parts contain the highest level of YncE protein (Figure 2). They were further selected for immunological analysis. Therefore, these two lines were used in the immunological analysis. The evaluation of the expression level in transgenic tobacco hairy roots was closely 0.9% of the TSP. A specific band of approximately 41 kDa corresponding to the YncE protein was verified by western blotting (Figure 3). Our findings showed that the proteins analysis extracted from the transgenic hairy roots with a level of expression for YncE was estimated to be 0.9% of the TSP.

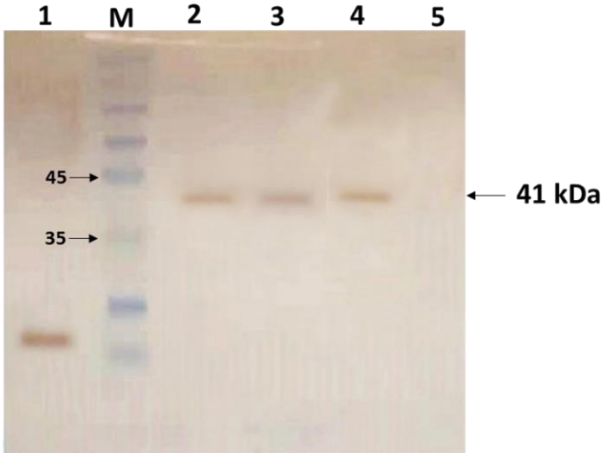
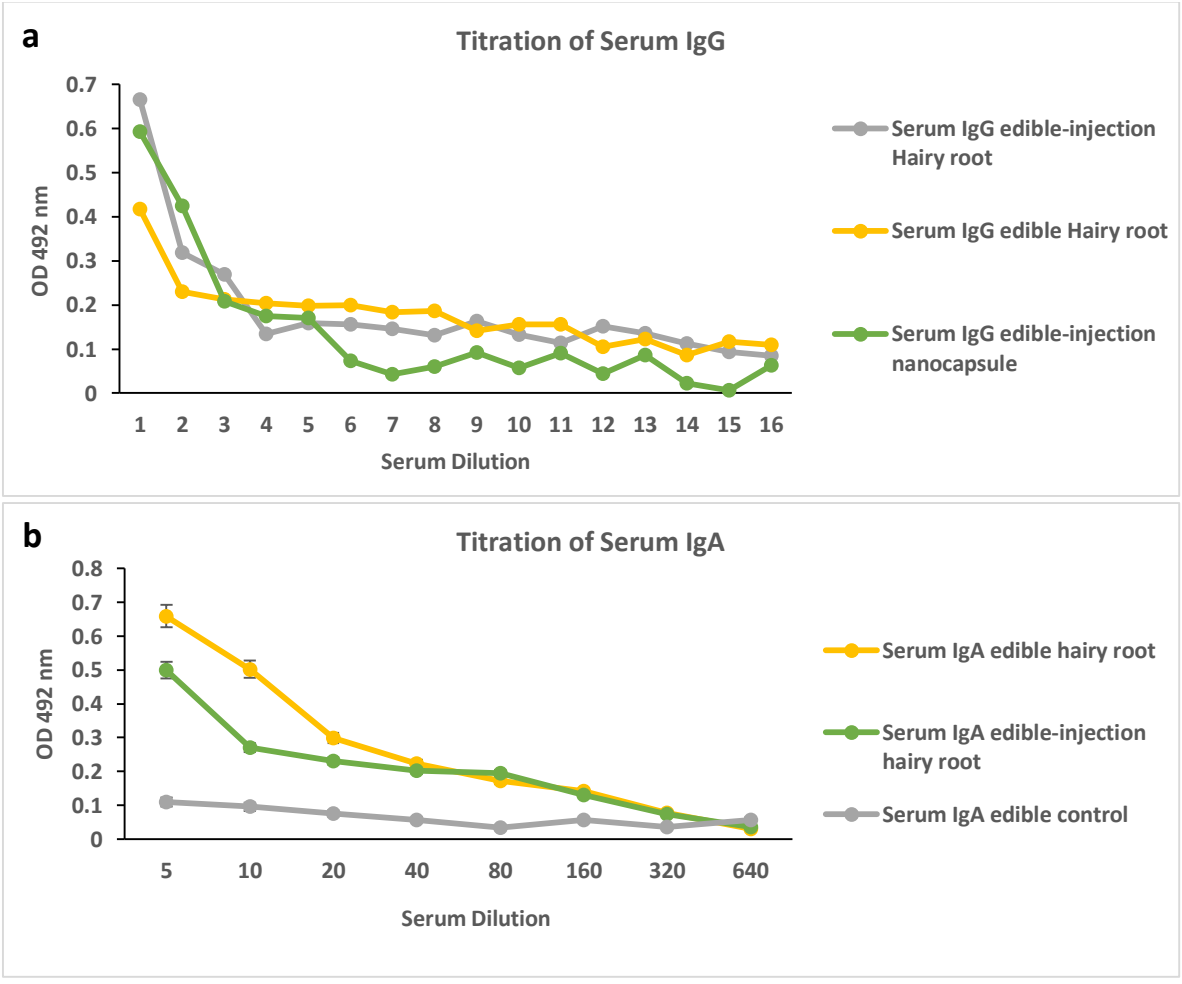


Figure 3. Western Blot Analysis Performed for 3 Best Lines of Transgenic Tobacco Hairy Roots Which Contain *YncE* Gene. Using anti histidine antibody. Line 1: Positive control (gatex protein 20 Kda). Line M: molecular weight markers. Line 2, 3, and 4: transgenic hairy root lines. Line 5: negative control (no transgenic hairy root).

Immunogenicity of Plant Derived YncE Protein in Mice

To determine the activity of antibodies, experiments were performed on three groups of mice (oral, oral-injection, and control). Humoral and mucosal antibody determination in serum and feces was performed by ELISA (Figure 4). The results showed that anti YncE IgG had the highest titration level of the antibody of the oral-injection group in the serum of mice (Figure 4a) but the results confirmed that anti-YncE IgA has the highest level of antibody titration in the serum of mice that received oral administration. (Figure 4b). Titration level of fecal showed that anti-YncE IgA is the highest titer in mice that administered edible hairy roots (Figure 4c). Titration of serum and fecal IgA in 3 groups showed that anti-YncE IgA had highest titration level in edible group compared two other groups (Figure 4d).



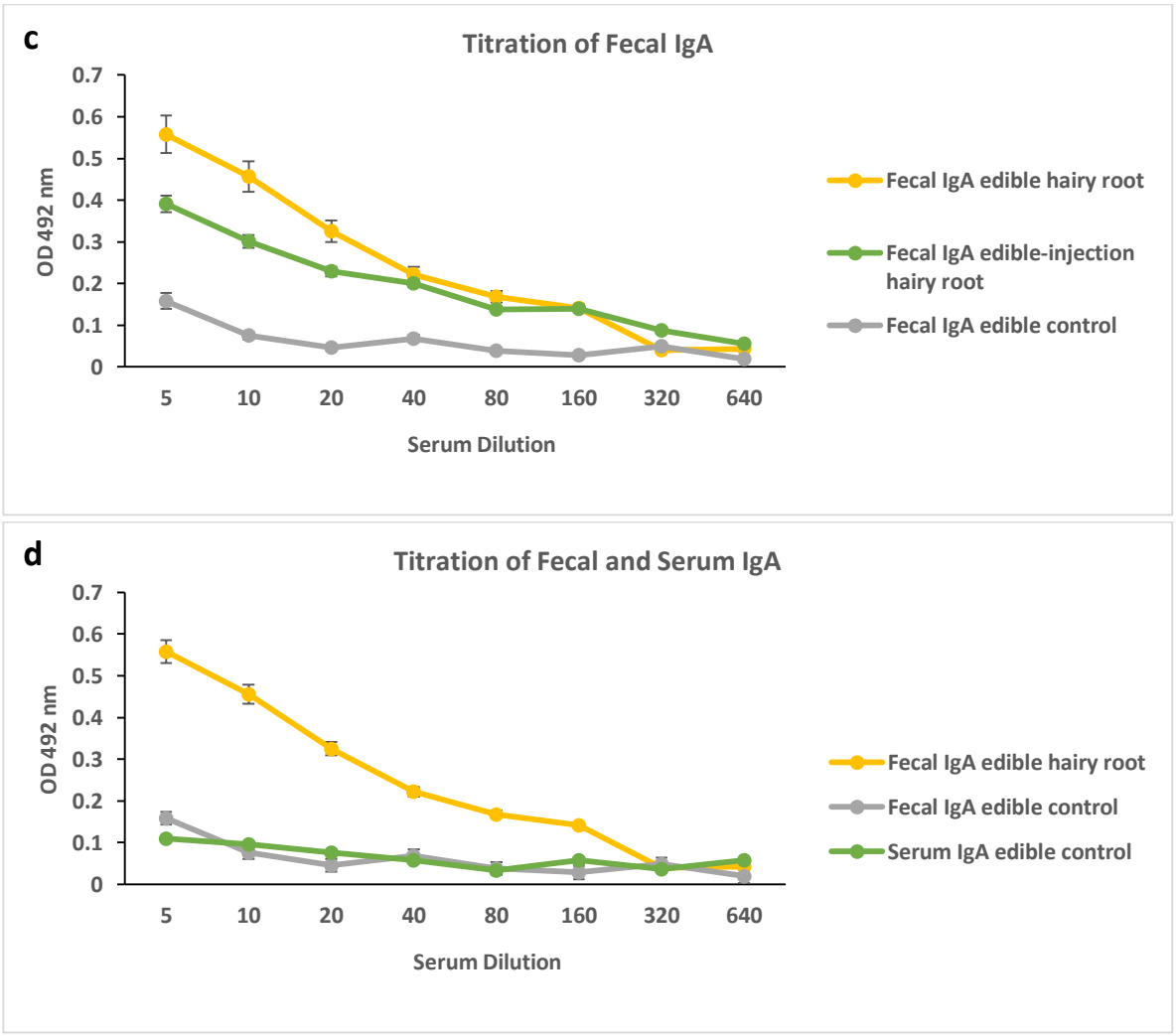


Figure 4. Determination of YncE Specific IgG and IgA by ELISA in Edible, Edible-Injection and Control Groups. **a)** Titration of serum IgG in 3 groups: 1. serum IgG edible-injection hairy root; 2. Serum IgG edible hairy root; and 3. Serum control injection. **b)** Titration of serum IgA in 3 groups: 1. Serum IgA edible- injection hairy root; 2. Serum IgA edible hairy root; and 3. Serum edible control. **c)** Titration of fecal IgA in 3 groups: 1. Fecal IgA edible-injection hairy root; 2. Fecal IgA edible hairy root; and 3. Fecal IgA edible control. **d)** Titration of serum and fecal IgA in 3 groups: 1. Fecal IgA edible hairy root; 2. Fecal IgA edible control; and 3. Serum IgA edible hairy root. Control group was administered with a soluble protein extract made from no transgenic tobacco hairy roots.

Discussion

Diarrheal diseases have been the third leading cause of death of children younger than five and are becoming an increasing burden in people aged 70 and over. The highest mortality from diarrheal diseases is in Sub-Saharan Africa and South Asia.[1] Sixty-two percent of tested cases were due to bacterial pathogens, with enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and Campylobacter predominating.[24] Enterotoxigenic *E. coli* (ETEC) is one of the most important strains of *E. coli* that causes diarrhea.[25,26] Vaccination is one of the most effective ways to control and prevent this disease.[27,28] The high costs and the difficulty of vaccination at a large scale are the two drawbacks in the path of wide utility of classical vaccines. Furthermore, live and inactivated vaccines are typically intended to provide a controlled diarrheal disease. Plant-based vaccines have many benefits. Edible-based delivery of vaccines has been generally considered as a lack of human and animal virus and the optimum route of vaccination owing to reliable immune response and reduced concerns over adverse effects of contamination with other pathogens. Another advantage is the correct fold and suitable post-translational modification and proper protein glycolysis. Hairy Roots (HR) provides an opportunity for the large-scale and low-cost production of subunit vaccines. Hairy systems appear to be among the most attractive in vitro culture systems because they combine the advantage of both suspension cell and whole-plant culture. They are the possible extracellular secretion of expressed proteins. In addition, the purification of recombinant proteins is easy. This approach was first used to produce a full length murine IgG1 antibody from tobacco HRs in 1997.[29] Oral vaccines stimulate the immune system in humans and animals and provide humoral and mucosal immunity.[24,30] Actually, 20 recombinant proteins have been

nearly expressed in HR systems. Examples of proteins expressed at high yields include acetylcholinesterase produced by *Nicotiana benthamiana* HR (3.3% total soluble protein)[31] and a 14D9 murine IgG1 produced by tobacco HR (64.03 mg/L).[32] Human IgG1 and IgG4 produced by tobacco HR (9.7-21.8 mg/g FW/d)[33] using a double-enhanced CaMV35S promoter (de35S) was developed to enhance transgene expression. In the current study, the HR method was used due to the fact that many studies reported mass production and better efficiency of recombinant protein in HR culture.[34] The YncE protein was accumulated at approximately 0.9% of TSP in HR under CaMV35S promoter control. In western blot analysis, a band of about 41 kDa using monoclonal anti-his HRP conjugate antibody was seen (Figure 3). The ELISA results showed that in serum IgG antibody of edible-injection, the hairy root group was higher than the other groups (Figure 4a). Furthermore, serum IgA antibody of orally hairy root group was higher than the edible-injection hairy root group and the control group (Figure 4b). The ELISA results showed that the fecal IgA antibody of edible hairy root was higher than the other groups (Figure 4c). Titration of fecal and serum IgA showed fecal IgA anti body higher than the other group (Figure 4d). On the other hand, the plant-based vaccine recombinant YncE protein could both have humoral and mucosal immune responses, effectively. Our results showed that the YncE antigen was able to stimulate the mouse immune system and produce antibodies. Actually, it appears that it could be a suitable vaccine candidate for ETEC-induced diarrhea.

Conclusion

Our present study revealed that YncE protein produced in transgenic hairy roots maintained its immunogenicity in mice after oral and oral-injection vaccination and is considered as a step towards the development of the expression of plant-made vaccine candidates as a low-cost biosimilar. We believe that more experiments should be conducted for the determination of the technical and economic feasibility of the implementation of hairy root-based vaccines.

Authors' Contributions

Study concept and design by FSA and JA; Analysis and interpretation of data by FML, MJM, and RK. Drafting of the manuscript and critical revision of the manuscript for important intellectual content by FML, FSA, and JA.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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