Expression of Heat-labile Enterotoxin of Escherichia coli in Biolistic Transformed Hairy Roots of Daucus carota L.

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ABSTRACT
Expression and secretion of recombination proteins in transgenic hairy roots have opened the door to simple, feasible and economic option compared to animal and bacterial systems. Here, we report a feasibility study for producing the nontoxic B subunit of Escherichia coli heat-labile enterotoxin (LTB) in Daucus carota L hairy root where transformation is achieved by biolistic gene gun method. Agrobacterium rhizogenes strain (R-1000) containing synthetic LTB gene in pMYO51T plant expression vector under the CaMV 35S promoter introduced to Daucus carota L roots by biolistic gene gun method. PCR amplification confirmed the integration of synthetic LTB gene in carrot hairy roots. Western blot analysis confirmed production of LTB pentamer in similar banding pattern to the native bacterial derived LTB. 0.50-0.70% of LTB protein estimated in carrot hairy roots. GM1-ganglioside assay indicated formation of biological active pentamers. LTB protein with biochemical properties identical to native LTB protein in the hairy roots of edible carrot roots opens the way for inexpensive, safe, and effective plant-based edible vaccines for oral delivery.7 Traditional vaccines are facing challenges like storage, distribution and administration but recently their safety and effectiveness has also raised many issues.2 Expression and secretion of recombination proteins, antigens and antibodies through transgenic hairy roots in confined media under controlled environment have opened door to simple, feasible and economic option compared to animal and bacterial systems.4,5 Hairy roots are non-photosynthetic and rapidly grow in simple culture media without requirement of phytohormones. Easy genetic manipulation, low-cost downstream purification and easy scale-up in bioreactors attract researchers to hairy roots.3 Heat-labile enterotoxin (LT) protein composed of one A-subunit (LTA, 27 kDa) and five non-covalently associated B-subunits (LTB, 11.6 kDa each) forming a ring-like pentamer from Escherichia coli causes diarrhea.3 Wild-type LT is toxic and therefore unsuitable for clinical use. Non-toxic LTB has high affinity towards the toxin receptor ganglioside GM1, a glycosphingolipid found ubiquitously on the surface of mammalian cells. Previous research confirmed that recombinant LTB could potential mucosal and parenteral vaccine adjuvants to stimulate strong serum and mucosal immune responses against LT.2 Nontoxic B subunit of Escherichia coli heat-labile enterotoxin (LTB) has been expressed in a number of plant species with favourable quantity, including potato (17 lg g-1 FW), tomato (38 lg g-1 DW), tobacco (75 lg g-1 FW) and carrot (3 lg LTB g-1 FW). However, present study reports biolistic gene gun mediated expression of LTB proteins in Daucus carota L hairy roots first time.

MATERIALS AND METHODS
Transformation vector
pMYO51T containing synthetic LTB gene (414 bp) controlled by the CaMV 35S promoter is used as plant expression vector.7 The recombinant plasmid was diluted 10 times, and 10 μL of this sample was used to transform 100 μL of Agrobacterium rhizogenes strain (R-1000) (at OD600 nm = 1) using the freeze-thaw method. A. rhizogenes strain (R-1000 ATCC) cells containing pMYO51T + synthetic LTB gene were added to YEB solid culture medium and activated three times at 27°C. A single colony isolated from the Petri dish was then inoculated into 25 mL YEB liquid culture medium and grown overnight at 27°C shaking incubator. This suspension culture is used for further experiments.

INTRODUCTION
Production of antigens and antibodies in plant provide a potential approach for the development of vaccines for oral delivery.7 Traditional vaccines are facing challenges like storage, distribution and administration but recently their safety and effectiveness has also raised many issues.2 Expression and secretion of recombination proteins, antigens and antibodies through transgenic hairy roots in confined media under controlled environment have opened door to simple, feasible and economic option compared to animal and bacterial systems.4,5 Hairy roots are non-photosynthetic and rapidly grow in simple culture media without requirement of phytohormones. Easy genetic manipulation, low-cost downstream purification and easy scale-up in bioreactors attract researchers to hairy roots.3 Heat-labile enterotoxin (LT) protein composed of one A-subunit (LTA, 27 kDa) and five non-covalently associated B-subunits (LTB, 11.6 kDa each) forming a ring-like pentamer from Escherichia coli causes diarrhea.3 Wild-type LT is toxic and therefore unsuitable for clinical use. Non-toxic LTB has high affinity towards the toxin receptor ganglioside GM1, a glycosphingolipid found ubiquitously on the surface of mammalian cells. Previous research confirmed that recombinant LTB could potential mucosal and parenteral vaccine adjuvants to stimulate strong serum and mucosal immune responses against LT.2 Nontoxic B subunit of Escherichia coli heat-labile enterotoxin (LTB) has been expressed in a number of plant species with favourable quantity, including potato (17 lg g-1 FW), tomato (38 lg g-1 DW), tobacco (75 lg g-1 FW) and carrot (3 lg LTB g-1 FW). However, present study reports biolistic gene gun mediated expression of LTB proteins in Daucus carota L hairy roots first time.

Plant material
One month laboratory grown carrot (Daucus carota L) roots were thoroughly cleaned with running tap water and disinfected by rinsing in 75% ethanol (v/v). The cleaned explants were finally treated with HgCl2 (0.1%) for 6–8 min under aseptic conditions and washed 5 times with sterilized distilled water to remove traces of HgCl2. Then, the surface sterile carrot root was sliced into several 0.5 cm thick discs and the cortex from approximately half of the pieces was further trimmed to expose the cambium and pre-cultured for 2 days on Murashige and Skoog (MS) medium added with 0.5 mg/L auxin before Agrobacterium transformation.

DNA bullet preparation
The gold particle stock was prepared by dissolving 60 mg of gold particles of size 1 μm provided by BioEra Life Sciences in 1 ml of 50 % sterile glycerol and vortexed for 5 min to avoid agglomeration of particles. The coating of plasmid DNA (0.5 μg) onto gold particles was carried according to manufacturers protocol (Bio-Era). This resulted in vortexing plasmid DNA and gold particles for 2–3 min in a solution of 20 μl of 0.1 M spermidine and 50 μl of 2.5 M CaCl2 followed by centrifugation at 5000 rpm for 5 min, collected pellet was rinsed twice subsequently in 150 μl of 70 and 100 % ethanol. The coated microparticles were then re-suspended in 50 μl of 100 % ethanol and kept on ice until bombardment. For each bombardment 10 μl of these microcarriers was used.9

Establishment of hairy roots
Pre-cultured carrot explants were bombarded with gold particles (1 μm) at 50 μg/μl concentrations coated with 2.0 μg/μl of pMYO51′ + synthetic LTB added A. rhizogenes culture using a Biolistic gene gun system (BioEra -Particle bombardment System) at 800 psi pressure and 6 cm flight distance. Further these materials were co-cultured on liquid MS medium at 25°C in the dark. After three days, the explants were transferred to fresh MS medium supplemented with 300 mg/L of cefotaxime and 100 mg/L of kanamycin and were maintained at 25°C under a 16/8 h light/dark photoperiod for two weeks. The developed hairy roots were subsequently transferred at two-week intervals to fresh MS agar containing antibiotics and incubated at 25°C in the dark. Healthy hairy roots were further analysed by PCR to confirm genomic DNA integration.

PCR analysis
Genomic DNA was extracted from the 2- week old hairy roots of carrot and screened by PCR to confirm presence of LTB sequence10 using specific primers for the LTB gene: Forward: 5′-GGATCCGCCACCATGGTGAAGGTGAAG-3′ and Reverse: 5′-GGTACCTCATAGCTCAT-CTTTC-3′. PCR products were subjected to electrophoresis on 0.8% agarose gel and stained with ethidium bromide.

Growth of hairy roots
The hairy roots confirmed to be transgenic were transferred to a 250-ml Erlenmeyer flask containing liquid MS medium without antibiotics and were grown at 28°C in the dark with mild shaking, refreshed medium weekly for two months for further experiments.

Extraction and purification of total proteins
The total proteins were extracted using phosphate buffer (100 mM, pH 7). First, the hairy root clones were ground under liquid nitrogen, and the powder was suspended in 1:1 phosphate buffer w/v. The recombinant protein was purified using low pressure chromatography system from GE Healthcare Life sciences TMN Buffer: Tris-MgCl2-NaCl buffer, shown 10–15 µg (0.75-1.2%) approximate amount of recombinant using SDS-PAGE, and ELISA immunodetection (BioEra Life Sciences) to confirm activity and specificity of the protein.

SDS-PAGE
The total proteins extracted from the transgenic and non-transgenic hairy roots were separated by 12.5 % SDS-PAGE.7,10 Further electrophoresis, the gel was stained using Coomassie brilliant blue, TMN Buffer: Tris-MgCl2-NaCl buffer,

Western blot analysis
SDS-PAGE separated protein bands were transferred from the gel to a Hybond C Extra membrane (GE Healthcare).7,10 Purified bacteria LTB (0.5 μg) were loaded as the positive control. Nonspecific antibody reactions were blocked by incubating the blot in 25 ml of 3 % (w/v) bovine serum albumin (BSA) in TBST buffer (TBS + 0.05% Tween-20) with gentle agitation overnight at room temperature. The blot was then incubated at room temperature for 2 h with gentle agitation in 10 ml of 1:2000 dilution of rabbit anti-LTB antisemur in TBST antibody dilution buffer containing 1.5% BSA and then washed three times with TBST buffer. Subsequently, the blot was incubated for 2 h at room temperature in a 1:5000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) in TBST buffer and washed three times with TBST buffer and once with TMN buffer. After washing, the colour was developed with BCIP/NBT in TMN buffer.

LTB-GM1 binding assay
The ability of LTB protein to bind to gangliosides is indication of immunogenicity.7,8,10 The microwell plate was coated with 100 μl/well of 3 μg/ml GM1 ganglioside (Sigma-Aldrich) in bicarbonate buffer, pH 9.6 at 4°C overnight. After three washes with PBST, the wells were blocked with 1% BSA in 0.01M PBS (300 μl/well) at 37°C for 2 h. The wells were washed three times with PBST and then incubated with the protein extract (100 μl/well) from the LTB transgenic carrot hairy roots for 2 h at 37°C. The wells were coated with 100 μl/well of 3.0 μg/ml BSA as a control. For the primary and secondary antibody treatments, the wells were incubated with a 1:5000 dilution of rabbit anti-LTB antibody (Sigma-Aldrich) (100 μl/well) in 0.01M PBS containing 0.5% BSA for 2 h at 37°C and washed four times with PBST. Subsequently, the wells were incubated with a 1:10000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich) (100 μl/ well) in 0.01M PBS containing 0.5% BSA for 2 h at 37°C and washed four times with PBST. Finally, the plate was incubated with100 μl/well TMB substrates (Sigma-Aldrich) for 30 min at RT in the dark. After incubation, the reaction was measured at an absorbance of 620 nm in an automated ELISA system (BioEra).

RESULTS AND DISCUSSION
Confirmation of transformation of Agrobacterium in carrot explants is done by important morphological markers i.e. proliferation of rapid growth, lateral branching and growth (Figure 1). Transgenic carrot hairy roots were successfully obtained. The integration of LTB gene in the carrot hairy roots is confirmed by polymerase chain reaction (PCR) (Figure 2). Western blot analysis of hairy roots carried out to confirm immunogenicity of the soluble proteins from the transgenic group against anti-LTB antisemur which revealed that specific signal from transgenic carrot hairy roots compared to no signal from untransformed roots. Results further confirm that transgenic carrot hairy roots are able to produce each oligomer of the LTB pentamer and show a similar banding pattern to the native bacterial derived LTB. Enzyme-linked immunosorbent assay (ELISA) was carried out to detect the antigen presence in the total soluble proteins from transgenic carrot hairy roots. Results of the ELISA and Western blot (Figures 3 and 4) showed 10–15 μg (0.75-1.2%) approximate amount of recombinant
plant-synthesized LTB protein per gram of fresh blotted hairy roots. The result showed that the soluble proteins from the transgenic group had immune reactivity against rabbit anti-LTB antiserum and the OD620 values of the transgenic groups were significantly higher than those of the negative controls which implied that LTB protein was expressed and accumulated in transgenic carrot hairy roots. LTB protein produced in carrot hairy roots demonstrated a strong relative affinity to GM1-ganglioside in the GM1-ELISA binding assays which also confirms LTB pentameric structure.

CONCLUSION

We conclude that nontoxic B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) proteins can be effectively expressed in transgenic carrot hairy roots and useful as an antigen source for a possible vaccine against diarrhoea. Research can be extended to in-vivo immune activity determination and scale-up of transgenic hairy root in suitable bioreactors.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

LTB: Nontoxic B subunit of *Escherichia coli* heat-labile enterotoxin, PCR: Polymerase chain reaction, YEB: Yeast Extract Beef media, BSA: Bovine Serum Albumin, BCIP: 5-bromo-4-chloro-3-indolyl-phosphate, NBT: Nitro Blue Tetrazolium, TBST: Tris-buffered saline, 0.1% Tween 20, ELISA: Enzyme-linked immunosorbent assay, GM1: monosialotetrahexosylganglioside.

REFERENCES


