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Expression of Cholera toxin B subunit in Banana callus culture

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Abstract:
Plants present a low cost agriculturally based effective production system for high value therapeutic system. The process of developing an edible vaccine begins by selecting a suitable vaccine gene of a pathogen and selection of plants to introduce vaccine gene in host. In the present study an attempt was made to produce edible plant vaccine against cholera expressed in Banana callus culture. *Vibrio cholerae* producing cholera toxins (CT) consists of two A (CT-A) and B subunits (CT-B), One subunit has 27 kDa (A subunit) which contain a toxic ADP ribosyl transferase activity and a pentamer of 11.6 kDa (B subunit). The CT-B subunit of *Vibrio cholerae* cells constituted an oral vaccine against cholera. In order to express CT-B protein in plant system, gene encoding cholera toxin B subunit (CT-B) was cloned into a vector pCAMBIA. CT-B gene was amplified by PCR and cloned into vectors pCAMBIA containing the strong, constitutive 35S CaMV promoter and a reiterated 35S enhancer. The plasmids were transformed into Banana callus via *Agrobacterium tumefaciens*. Production of CT-B vaccine in banana plant callus for expression and delivery of edible vaccines. Banana callus proteins were analyzed for the confirmation of recombinant CT-B. Transgenic plants expressing CT-B showed the presence of a protein that was recognized by mouse anti CT-B antibody and which migrated to the same position in SDS-PAGE as CT-B derived from *Vibrio cholerae*. Edible vaccines against *Vibrio cholerae* has expressed in Banana which could be consumed raw in the form of fruit.

Key words Antigen, CT-B, *Robusta sp,* Transgenic plants, *Vibrio cholerae.*

Introduction:

Vaccines have accomplished near miracles in the fight against infectious diseases, it play a key role in healthcare. However, the cost of production and maintaining a chain for vaccine distribution has so far hampered realizing their full potential. The development of plant biotechnology has promoted the scientists to express foreign antigens in plant tissue as edible vaccine vehicles [1]. Expression of antigens to be used as vaccines, against of pathogens in transgenic plants is a convenient and inexpensive source for these immunotherapeutic molecules.

In addition, developing safe vaccines has been another important objective in vaccine research since conventional vaccines having viable attenuated microbial components pose small risks to the world population [2]. For this reason, vaccine makers today favor subunit vaccines that contain primarily the antigenic epitopes from pathogens as vaccine component instead the whole attenuated live or killed pathogens in the vaccines. These antigenic epitopes have no way of establishing an infection but offer production against infectious pathogens.

Cholera is an acutely dehydrating, watery, diarrheal disease caused by intestinal infection with the bacterium *Vibrio cholerae*. Among the multiple virulence factors in *Vibrio cholerae*, Cholera toxin (CT) plays a major role in the pathogenesis of infection. Oral vaccination can lead to protection against entering the body via mucosal surfaces of the host [8]. The enterotoxin LT from *Escherichia coli* is a multimeric protein, quite similar to Cholera toxin (CT) structurally, functionally and antigenically. LT has one A subunit (27kDa) and a pentamer of B subunits (11.6kDa). Binding of the non toxic LT-B pentamer to GM1 gangliosides, present on epithelial cell surfaces, allows oral immunegene. The A sub unit (CT-A) is an NAD-dependent ADP ribosyl transferase that responsible for cholera [5].

An oral vaccine composed of the cholera toxin–B subunit (CT-B) with killed *Vibrio cholerae* cells has been reported to give significant level of protection against Cholera. But the cost of production of CT-B by conventional methods is too high to allow distribution of this vaccine [11]. Oral vaccines have the advantage of being safe

and easy to administer but exploitation is hampered by low efficacy, induction of immuno tolerance rather than an immune response.

Proteolytic form of antigen is degraded during passage through the gastro-intestinal tract and exposure to extremely acidic conditions in the stomach [10]. Naturally stable and encapsulated antigen may survive the harse environment of the gastric intestinal tract. The relatively strong immuno genicity after local administration might be related to the instrinsic mucosal adjuvant activity of these components [6].

In the present study, an attempt was made towards the production of edible vaccine by expressing CT-B subunits of cholera toxin in Banana callus culture, through *Agrobacterium* mediated gene transfer methods. Transgenic plants are capable of producing several different products can be created at any given time by crossing plants producing different products. Plants are being looked upon as potential bioreactors of bio-factories for the production of immunotherapeutic molecules.

Materials and Methods:

Growth and Maintenance of Strains, Plasmids and media: *Vibrio cholerae* strain 569 B was used for amplification of ctx B gene. *Vibrio cholerae* was grown in Luria bertani medium (Casein 1%, Yeast o.5%, Sodium chloride 1%) containing Rifampicin (30 g/ml) antibiotic for 24 hr incubation. *Agrobacterium tumefacions* (LBA4404) was grown in YPS medium (Yeast extract 1%, Peptone 1%, Sodium Chloride 0.5% at pH 7.0) containing 50 µg/ml Kanamycin at 28˚C for 24 hr.

Construction of cloning vector Encoding CT-B

Escherichia coli DH5α (stratagene) PRK2013 together with pBluescript II KS were used for the initial cloning, sequencing and maintenance of different DNA fragments. The *Escherichia coli* were grown on LB medium with 50µg/ml Kanamycin [7]. For recombinant protein production, all the products were initially cloned in PGEM-T. Easy vector (invitrogene) and later using the unique restriction sites available in the existing sequence, they were assembled to PGA 643 a shuttle vector was used. All the chemicals and enzymes were obtained from Merck and Fermentase companies, respectively.

Construction of expression vector encoding CTB

General DNA manipulation was performed using typical recombinant procedures [7]. DNA fragments were analyzed by electrophoresis on a 0.8% (w/v) agarose gel and purified from the agarose gel by, gel extraction kit according to the manufacture's recommendation.

Oligonucleotide primers: $^{5'}$TATGGATCCATGACACCTCAAAATATTACT$^{3'}$and $^{5'}$GGCGAATTCATATCTTAATTTGCCATAC$^{3'}$ were designed according to the published sequence for CT-B. PCR was carried out (Thermal cycler) to create Bam HI- EcoRI ctx B cloning cassette. The following conditions were used for amplification: hot start at 95^{o} C for 5 min, followed by 30 cycles of denaturation at 95^{o}C for 1 min, annealing at 55 ^{o} C for 1min and extension at 72^{o} C for 1min. The program was followed by a final extension for 5 min. The amplified ctxB cassette was confirmed by digested with both Bam HI and EcoRI and recovered in pBluescript KS II. PCR fidelity was verified by complete sequencing of the ctx B portion. The cassette was then excised and sub cloned between BamHI and EcoRI sites of PGA 643 to create plant expressing plasmid PCAMBIA.

Expression of CT-B antigen gene in Plants via stable Transformation

Genetic Transformation of plants has facilitated the study of plant gene expression and CT-B foreign proteins have been

successfully expressed in plants. Transformation can be carried out using *Agrobacterium* T-DNA vector, high levels of expression can be achieved by using strong specific plant promoters. CT-B gene was amplified by PCR and cloned into vector containing the strong, constitutive 35S CaMV promoter and a reiterated 35S enhancer. The plasmids were transformed into Banana callus via *Agrobacterium tumefaciens*.

SDS PAGE & Western Blot Analysis:

Protein was extracted from callus tissue as described [4].The total soluble protein from transformed and non transformed cells was separated on a 12% SDS-PAGE [3] and coommassive brilliant blue stained. Sample was used for western analysis with anti-cholera toxin monoclonal antibodies was used as a primary antibody and rabbit anti-mouse IgG peroxidase conjugate (sigma) as a secondary antibody (2:20,000dilution). The reaction was developed by DAB (Diaminobenzidine-H_2O_2) solution (Sigma). Western blot was carried out as described [7].

Results and Discussion:

In this study, CT-B gene was cloned into plant expression vector. The plasmid PCAMBIA [Fig 4] was constructed by inserting the coding region for CT-B from PRK2013 together with pBluescript II KS [Fig 2] between the Bam H1 and EcoRI in the sites of PGA643 plant transformation vector and expressed in the plasmid (PCAMBIA). This system was used to transform recombinant gene in banana by the microinjection method using *Agrobacterium tumefaciens*, and regenerated callus culture were analyzed by western blot analysis. Protein was extracted from callus tissue and the total soluble protein from transformed [Fig 5] and non transformed cells was separated on a 12% SDS PAGE [3] and coommassive brilliant blue stained.

The total soluble proteins were extracted from 1g of callus tissue. The resulting extract was concentrated to 100µl by freeze drying in low speed in a high vacuum. 20µl of the above sample was used for western analysis [Fig 6]. Western analysis confirmed the presence of CT-B antigen specific band. The results revealed that the denatured CT-B expressed in plant cells was showed 11.6 kDa protein bands similar to CT-B derived from *Vibrio cholerae*.

Banana callus expressing CT-B showed [Fig 5] the presence of a protein that migrate to the same position in denaturing gel as the CT-B derived from *V. cholerae* and was recognized by mouse anti CT-B antibody. Cholera toxin B subunit, when expressed in plant, was processed in a natural way. The pentameric form being the abundant form. Antigenically it was found to be similar to the bacterial protein. Even after boiling transgenic products till they became soft, approximately 50% of the CT-B was present in the pentameric GM1, ganglioside-binding form.

PCR analysis of CT-B gene in banana callus

Integration of the transgene was confirmed by PCR using genomic DNA isolated from transformed and control callus/cells. DNA was isolated by CTAB method essentially as described [9]. The following two primers were used to amplify 256 bp fragment of CT-B gene.

Oligonucleotide primers: $^{5'}$TATGGATCCATGACACCTCAAAATATTACT$^{3'}$and $^{5'}$GGCGAATTCATATCTTAATTTGCCATAC$^{3'}$ were used to carry out PCR reaction. The recovered plasmid was further analyzed by PCR to confirm the presence of CT-B cassette in the recovered plasmid by 0.7% agarose gel electrophoresis shown in Figure 3.

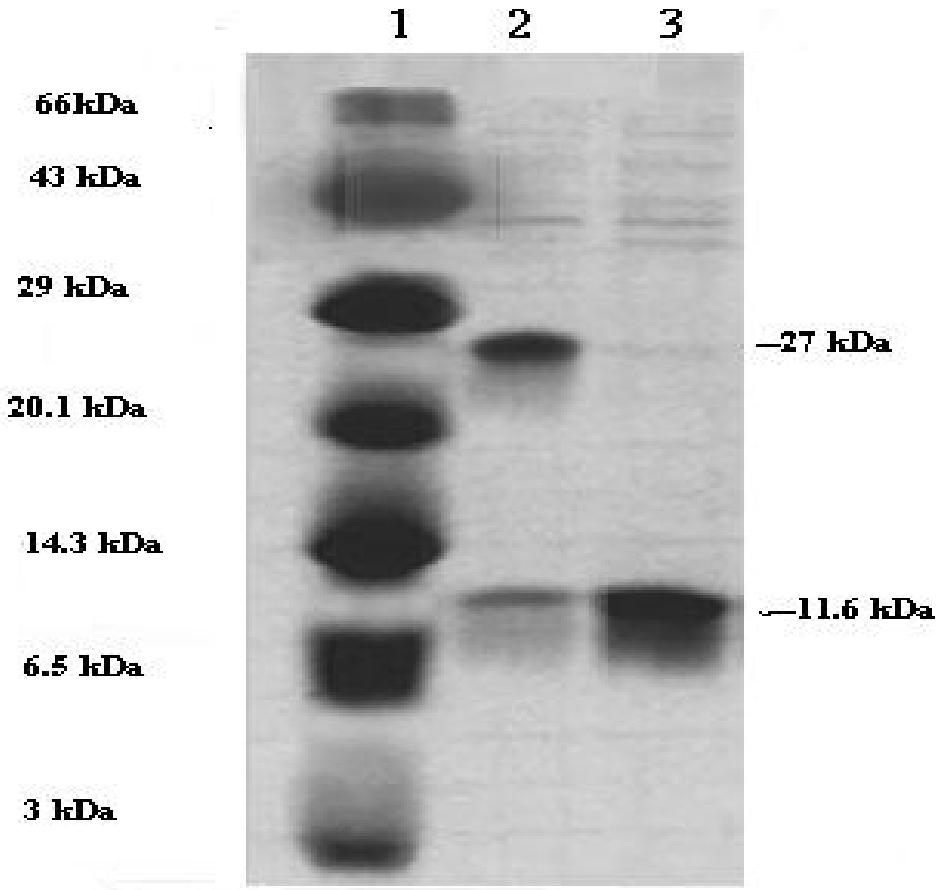

Figure 1: SDS-PAGE showed the presence of cholera toxin

Lane 1 - Standard protein marker, Lane 2 -Isolated cholera toxin showed 27 kDa (CT-A) and11.6kDa (CT-B), Lane 3 -Elution of pure CT-B confirmed its size as 11.6 KDa

DNA from Kanamycin resistant cell lines/ callus was isolated and confirmed for transgenic nature by PCR. A diagnostic 256 bp fragment amplified with primers specific to CT-B gene was detected transformed banana callus. The transformed Banana callus cells showed an 11.6 kDa size band corresponding to CT-B, but not in the non transformed cells of banana.

Confirmation of CT-B antigen gene in banana (Robusta sp.) callus

The CT-B encoded protein (10 µg) was injected in to the 3 months old callus of banana species [Fig 7a] by micro syringe method and the callus was maintained in the same culture chamber under aseptic conditions provides with light intensity and temperature control. Subsequent subcultures were maintained at regular time interval. After 4 months of it s growth, 5 mm size of callus segment were separated from the mother culture and tissues homogenized which containing CT-B expressed product was prepared from homogenized mixer. Then extracted CT-B product can be used for its confirmed expression in banana culture by western blot analysis [Fig 6].

Conclusions:

Cholera was an acute diarrhea infection caused by ingestion of the bacterium *Vibrio cholerae*. Disease was characterized in its most severe form by sudden acute watery

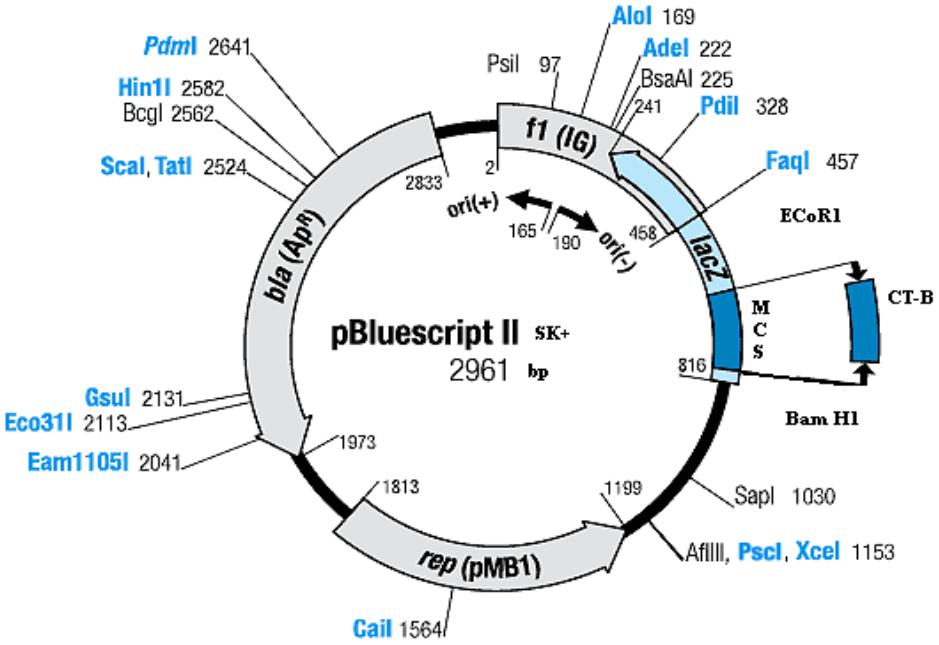

Figure 2: pBLUESCRIPT SK+ with CT-B gene

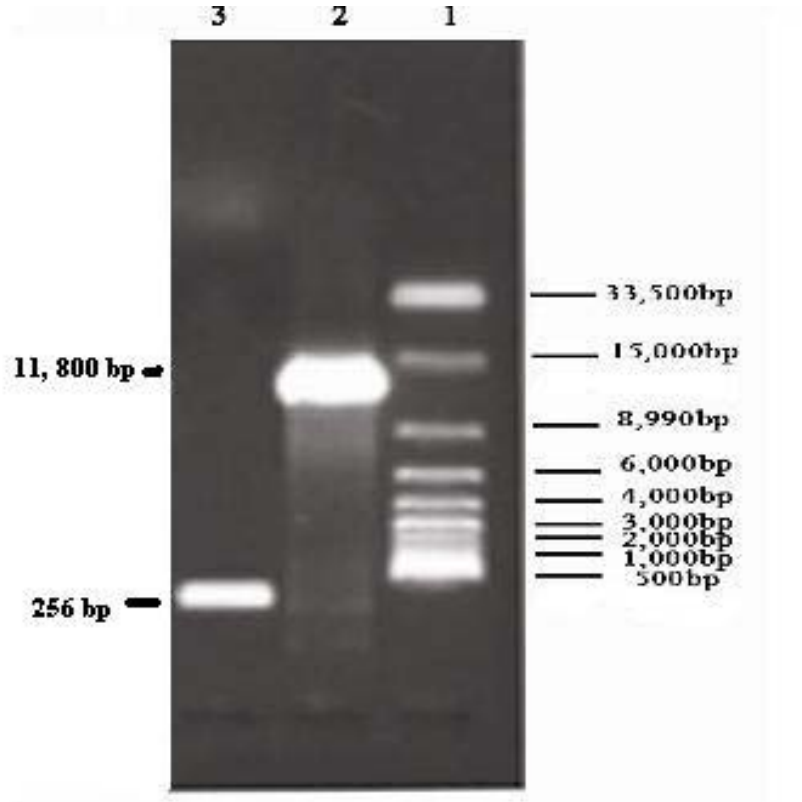

Figure 3: PCR amplification of CT-B from *Vibrio cholerae* and eluted PCR product

Lane 1- Molecular weight markerSuper mix DNA, Lane 2- Ti plasmid (11, 800 bp), Lane 3- amplified CT-B antigen gene (256 bp)

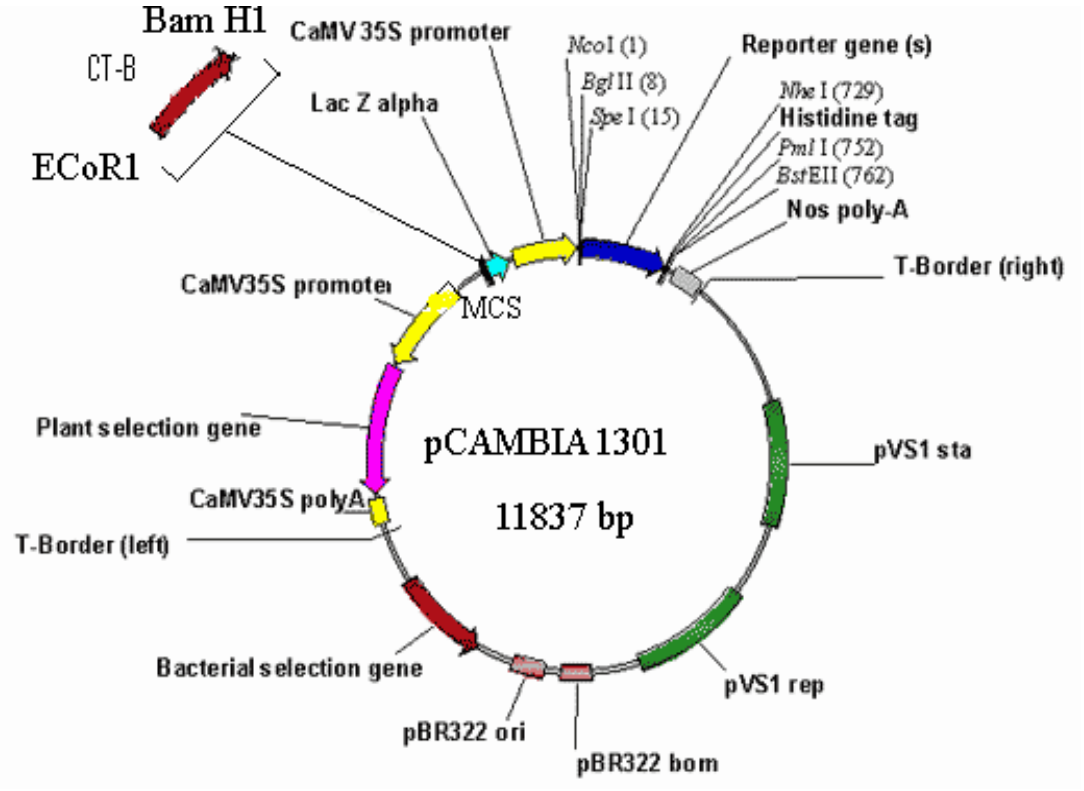

Figure 4: Construction of p CAMBIA 1301 containing CT-B gene

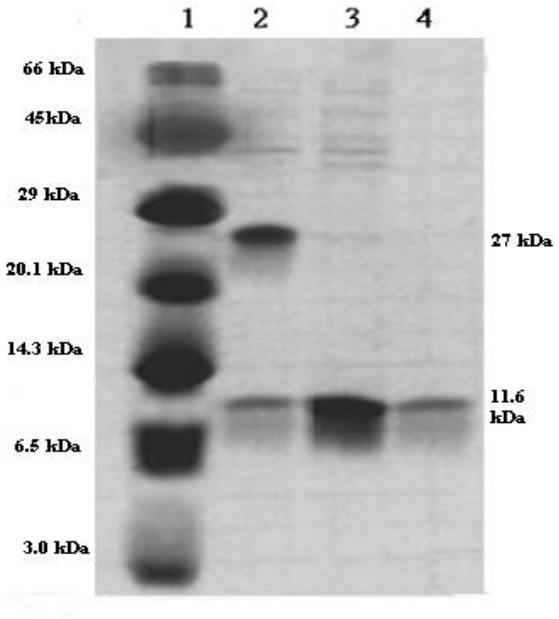

Figure 5: Expression of recombinant CT-B

Lane 1- Standard Protein marker, Lane 2- Cholera toxin from *V. cholerae* consists 27 kDa (CT-A) and 11. 6 kDa(CT-B), Lane 3- After elution of CT-B from SDS-PAGE showed presence 11.6kDa., Lane 4- CT-B antigen after transformation indicates single band at11.6kDa.

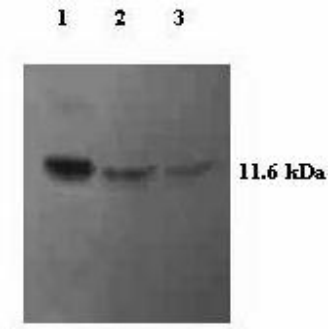

Figure 6: Western blot analysis of CT-B in transgenic callus of banana
Lane 1- Control Cholera toxin CT-B
Lane 2- 25 µg of cholera toxin from 3 month grown callus after injection of CT-B
Lane 3- 25 µg of cholera toxin from 6 month grown plantlet culture after injection of CT-B)

Figure 7 a: Banana plantlet emerged from callus culture
Three month grown transgenic plantlet of Banana [Robusta sp.]

Figure 7 b: Transgenic Banana plants
Six month grown Banana plantlet was separated individually without damaging roots of a plant cell. MS medium was removed by washing with sterile distilled water after that plantlets was ready to garden in the green house.

diarrhea that can lead to death by severe dehydration. Vaccines have been accomplished near miracles in the fight against infectious diseases. Vaccination was the best known and most successful application of immunological principles to human health. The CT-B antigen was prepared from *Vibrio cholerae*. The size of the CT-B antigen (11.6 kDa) was confirmed by 12% SDS-PAGE [Fig 1] experiment. Then CT-B antigen was eluted from SDS-PAGE and then used for vector construction. The expression of CT-B antigen was confirmed by amplification of product by polymerase chain reaction (PCR). PCR was carried out to create Bam H1 and EcoRI in CT-B cloning cassette. The CT-B cassette was digested with both Bam H1 and EcoRI then recovered in pBLUESCRIPT SK+. The cassette was then excised and sub cloned gene between the Bam H1 and EcoRI sites of pGEM-T to create a clone contains the CT-B gene in the callus was allowed to grow in MS medium. The subculture was maintained at regular interval until to differentiate the callus in to plantlets. The presence of CT-B expression in Banana culture was confirmed by western blot analysis. The result revealed the presence of 11.6 kDa CT-B antigen in transgenic callus culture was experimentally confirmed transformed gene expressed it product in the analysis of Banana (Robusta species) Callus. The remaining callus was allowed to grow in the same experimental condition to differentiate in to multiple shoot, subsequently developed in to plantlets of Banana from the same callus [Fig 7b]. The young plantlets were separated from the mass of MS medium culture and transplanted in the garden soil, then allowed to grow under green house. CT-B was one of the bacterial antigens expressed in edible plants. In this present study we have selected Banana (Robusta sp.) to express the recombinant CT-B antigen because, it was a

well accepted fruit and could be eaten as a raw fruit. Another reason, it would be grown all the parts of the world.

The development of plant biotechnology was promoted to express the foreign antigen in plant tissues as edible vaccine. The Oral Cholera Vaccine (OCV) therapy needed more cost for immunization against cholera, but edible vaccines were very economic and less cost consuming therapy against cholera.

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