Transgenic Banana callus derived recombinant cholera toxin B

Subunit-as potential vaccines

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Abstract

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) which has 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. The production of a vaccine in plants depends upon the availability of a DNA sequence coding for a protective antigen and the construction of an expression system suitable for plant transformation. The new construct was transformed into culture of banana (Robusta sp) plant callus cells. SDS-PAGE and immune blotting methods were used to confirm the expression of CT-B in callus. The use of plant derived vaccines may overcome some of the major problems encountered with traditional vaccination against infectious disease. Banana are good candidates for edible vaccine as they are eaten raw inexpensive to produce and native to many developing countries.

Keywords: CT-B, ELISA, Vibrio cholera, Robusta sp, western blot analysis

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Introduction

Recombinant DNA technology has already radically altered the field of vaccines. Molecular biology also facilitates the development, production and delivery of safe and effective vaccines. Vaccines are primary tools in programmers of health intervention for both humans and animals. Vaccines have been revolutionary for the prevention of infectious diseases. During the past decade, advances in molecular immunology have led to development of effective vaccination programmers. However, the cost of production of vaccines by traditional fermentation based systems is high and is hampering progress in this direction. Creating edible vaccines involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. This process is known as transformation and the altered plants are called transgenic plants (Lal et al., 2007). Subunit vaccines are commercially produced in genetically engineered bacteria, yeast, and plant. The quantity of plant tissue that may constitute a vaccine does must be of practical size both for field production and for consumption.

Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of pathogenic genes. Thus, they have no way of establishing infection assuring its safety, especially in immune compromised patients. The goal is to produce plant organs (leaves, fruit) crude extracts (dry protein powder) or
purified power) or purified proteins that upon oral or potential administration deliver one (or more) immunogenic protein(s) in a manner that triggers an immune response (Kulkami et al., 2006). The applications of plants as protein production systems are wide and varied. The first demonstration of expression of a vaccine antigen within plants occurred in 1990 when Curtiss and Cardineau (1990) expressed the Streptococcus mutans surface protein antigen A (Spa A) in tobacco. This demonstration was closely followed by plant expression of the hepatitis B surface antigen (Kapustin et al., 1999), the E.coli heat labile enterotoxin responsible for diarrhea (Walmsley and Arntzen, 2000), the Norwalk virus capsid protein and the rabies virus glycoprotein (Mc Garvey et al., 1995). Proteins produced in these plants induced synthesis of antigen specific mucosal Ig A and Ig G when delivered orally to mice and humans. However, the focus within this paper will be restricted to plants derived therapeutics for active immunization.

An oral vaccine composed of the cholera toxbin-B subunit (CT-B) with killed V. 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. In the present study, an attempt was made towards the production of edible vaccine by expressing Ct-B subunit of cholera toxin in Banana callus culture through Agrobacterium mediated gene transfer techniques.

**Material and Methods**

Bacterial strain and growth media: Vaccines contain DNA fragment from Vibrio cholerae strain 569 B. These fragments code for a protein that is usually a surface protein of the pathogen. This is responsible for eliciting the body’s immune response. Vibrio cholerae was grown in Luria bertani medium (casein 1%, yeast 0.5%, sodium chloride 1%) containing rifampicin (30 g/ml) antibiotic for 24 hr incubation. Agrobacterium tumefaciens (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 hrs. Gene (ctx B) encoding antigen from Vibrio cholerae that have been amplified, epitope within the antigen are identified, DNA fragment encoding these can be used to construct genes by fusion with a coat protein gene from CaMV and the recombinant strain was used to infect stabilized callus, the resultant protein are utilized for further immunological studies.

**Plant expression vector**

*Escherichia coli* DH5α (stratagene) PRK2013 together with pBluescript II KS were used for the initial cloning, sequencing and maintenance of different DNA fragments. The *E.coli* was grown on LB medium with 50 µg/ml Kanamycin (Sambrook et al., 1989). For recombinant protein production, all the products were initially cloned in PGEM-T. Easy vector (Invitrogen) 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.

The ctxB gene was cloned by PCR with primers using chromosomal DNA prepared from Vibrio cholerae strain 569 B as the DNA template. Oligonucleotide primers:

\[5^{'}TATGGATCCATGACACCTAAAATATTACCT^{3{'}} \]

\[5^{'}GGCGAATTCTATCTTTAATTTGCCATAC^{3{'}} \]

were designed according to the published sequence for CT-B. PCR was carried out to create *Bam HI-EcoR1* CtxB cloning cassette. The following conditions were used for
amplification: hot start at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 5 min. 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 ctxB portion. The cassette was then excised and sub cloned between BamHI and EcoRI sites of PGA 643 to create plant expressing plasmid pCAMBIA. 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 35SCaMV promoter and a reiterated 35S enhancer. The plasmids were transformed into Banana callus via Agrobacterium tumefaciens. Banana calluses were developed as shoots on MS medium supplemented with 0.05 mg 1-1 of indole acetic acid, 7 g 1-1 of agar, 100 mg 1-1 of kanamycin, 250 mg 1-1 of cefotaxin. Regenerated shoots were segregated and sub culture in fresh medium and transgenic plants were multiplied in vitro.

Transgenic callus were initially selected on the basis of resistance to the presence of the CT-B gene was confirmed by PCR analysis. Integration of the transgene was confirmed by PCR using genomic DNA isolated from transformed and control cells. Plant DNA was extracted from callus as follows: 0.2 g of callus was collected in a 1.5 ml eppendorf tube and ground with a plastic pestle in 400 μl extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS). An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added and mixed well by inverting the tube several times. The tubes were centrifuged at maximum speed on a bench top centrifuge for 10 min. The aqueous phase was transferred to a clean eppendorf tube and the DNA precipitated with 2-propanol and washed with 70% ethanol. PCR was carried out successfully to create a gene fragment encoding the sequence of mature CT-B. The eluted PCR product was analyzed by digestion with Taq-1, the presence of CT-B was confirmed by PCR following the plasmid recovery of the CT-B cassette in the transformed banana but not in the non transformed callus culture.
**Analysis of protein from banana callus**

Protein was extracted from callus tissue as described earlier (Mason et al., 1992). The total soluble protein from transformed and non transformed cells was separated on a 12% SDS-PAGE (Laemmli, 1970) and silver stained. Total soluble proteins were extracted from 1 g of callus tissue and the resulting extract was concentrated to 100 by freeze drying in low mode in a speed vac. The soluble protein was re-extracted into 20 mM phosphate buffer pH 7.4 using centricon columns (Amicon) and 20 µl of the sample was used for western analysis. 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 (2:20,000 dilution). The reaction was developed by DAB (diaminobenzidine-H₂O₂) solution (Sigma). Western blot was carried out as described by Sambrook et al. (1989).

**ELISA quantification of CT-B**

The amount of banana total soluble protein content was estimated by a Bradford assay (Hurst, 1991). Comparison of total soluble protein of the induced and non induced banana cells were used to estimate the recombinant CT-B expression levels. The extract of banana callus cells contains proteins that complicate the assessment of the recombinant protein, especially when the recombinant protein level is low. SDS-PAGE analysis of TSP (boiled and unboiled) showed only a thin band of recombinant CT-B in the expected position. Thus, to facilitate evaluation of the recombinant protein in the immuno-blotting assay, the banana total soluble protein was partially purified and used for SDS-PAGE and immune blotting analysis were used for evaluation of the presence of the presence and antigenicity of the new recombinant protein. In order to determine the antigenicity of the recombinant CT-B, western blot analysis of TSP was performed. Total protein concentration of the plant samples was determined using coomassie dye binding assay (BioRad), using bovine serum albumin (BSA) as a standard.

**Results and Discussion**

Developing embryogenic culture systems with reliable regeneration efficiency from *Robusta* sp of banana is a prerequisite for realizing the potential of cellular and molecular tools of crop improvement. The original explants became brown at the base within a week of culture and began to swell and the size also increased after 2-3 weeks, when the embryogenic callus induction was highest in banana which upon transfer to suspension medium friable embryogenic callus released embryogenic cells with dense cytoplasm, suspension cultures obtained consisted of heterogenous cells (Fig. 1).

**Fig. 1. Development callus of banana explant**

The cultivars exhibited different response in term of the time taken for callus proliferation embryo development and embryo conversion to plants. Somatic embryo emerged into the plantlets on 1/2 strength MS basal medium with 0.1% activated charcoal and 0.2 + gelrite and the time span for the development of shoot and root was within 7 to 9 weeks. In banana for the initiation of callus medium is supplemented with three auxins IAA, NAA, 2,4-D and
different auxins for the proliferation while cytokinin with (IAA+BA) is necessary for embryo development. Genetic manipulation using embryogenic cell suspension has become a tangible and useful approach for an integrated genetic improvement via Agrobacterium mediated gene transfer of CT-B encoded gene expressing antigen in the transgenic plants (Fig. 2). Once the plantlets developed these were separated and transplanted into individual container (cups) for further development as regenerative potential for use in propagation and genetic improvement.

**Fig. 2.** Plantlet emerged from callus

Transgenic Banana vaccine engineered to express cholera toxin B subunit analogous to the closely related LT-B, induced both serum and intestinal anti CT-B antibodies after being fed to mice in different doses (Data was not shown). Gene encoding antigen from pathogenic organisms that have been characterized and for which antibodies were available, the entire structural gene is inserted in to a plant transformation vector between 5’ and 3’ regulatory elements which could allow transcription and accumulation of coding sequence in plant. The data presented in this paper prove that CT-B antigen gene could be expressed in banana efficiently. Transgenic plants expressing recombinant antigens have been developed successfully since the method was first described by mason et al. (1992). Plants are recognized as safe and cheap production system for proteins of pharmaceutical interest including vaccines. Recombinant plants expressing antigens or antibodies have been developed successfully by using Agrobacterium tumefaciens mediated transformation.

In this work examined the production of rec CT-B in banana callus and the applicability of the callus for oral immunization. In order to obtain sufficient production levels of this bacterial protein in transgenic plants, as amplified gene was made for retention in the system.

**Fig. 3.** 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 11.6 KDa, Lane 4-CT-B antigen after transformation indicates single band at 11.6 KDa)

Banana callus expressing CT-B showed (Fig. 3) 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. The total soluble proteins were extracted from 1 g of callus tissue. The resulting extract was concentrated to 100 µl by freeze drying in low mode in a speed vac. 20 µl of the above sample was used for western analysis (Fig. 4). 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 cholera.

Fig. 4. 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).

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 sp 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 its growth, take 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 ELISA blot analysis (Fig. 5). Results showed that the Maximum expression levels of CT-B in 0.9 µg g⁻¹ f.wt of callus in transformed cell line were observed. In transgenic banana plants maximum expression level of 0.7 µg g⁻¹ f.wt of leaf of CT-B was noted in emerged plantlet. The transformed plants hardened in the green house showed moderate expression level of 0.5 µg g⁻¹ f.wt of CT-B.

CT-B could also be secreted into medium by callus cells in the required particulate form as evidenced by ELISA results. However, the level of secretion was low compared to intracellular accumulation as no secretary signal was fused to the CT-B gene. The production of recombinant proteins in cell suspension cultures is still as an early stage the results suggest that this can be scaled up further to produce CT-B in large quantities under controlled conditions. Plants generally produce relatively small amounts of recombinant protein which correlates with potential limitations in the amount of antigen that can be introduced by direct consumption. Banana producing CT-B were offered a dose similar to that used for oral immunization against a purified cholera B subunit (CTB), an enter toxin protein that is similar in structure and immunological cross-reactivity to LTB. Figure 5 revealed the confirmation of CT-B expression under RNA blot analysis could be explained under proper transcript mechanism. It seems that the amount of the CT-B produced in plant cell is enough to elicit immune responses. It may induce mucosal and Banana systemic anti-cholera toxin antibodies at the levels sufficient to provide protective immunity against the cholera toxin. Approximately 1.0 mg of CTB per oral dose is required for
protection against cholera induced diarrhea (Clements et al., 1990). Therefore, at a 0.1% expression level, 50 to 100 g of transgenic raw, Banana was needed for an equivalent dose (~ 2% protein).

**Fig. 5.** ELISA analysis of Transgenic Banana culture (X-Developing stages of Banana culture indicates c-callus; EP-Emerged Plantlet; DP-Fully Developed Plantlet; MP-Mature Plant. Y-Expression of CT-B in terms of µg/g fresh wt. culture)

The employment of edible transformed plants as production and delivery vehicles for immunogenic peptides is providing the basis for a dramatic breakthrough in vaccine protective efficacy. Most plant genomes can accept large gene inserts without disrupting normal plant functions. Because plant genomes usually contain large numbers of multi copy genes, an insertion into one copy frequently does not affect the health of the plant. Numerous genes can be introduced into a single plant and several different transgenic plants can be cross-pollinated to produce multimeric proteins or multiple antigenic epitopes for broad-spectrum vaccination. Plants also carry out post translational modification of newly synthesized proteins, which frequently retains the biological activity of the foreign protein. The autotrophic nature of plants allows them to synthesize proteins with medicinal value for the relatively small cost of soil, water, and harvesting labor.

**Conclusion**

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 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 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 *EcoR1* in CT-B cloning cassette. The CT-B cassette was digested with both *Bam H1* and *EcoR1* then recovered in pBLUESCRIPT SK+. The cassette was then excised and sub cloned gene between the *Bam H1* and *EcoR1* 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* sp.) 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. 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.

References


