Synthesis and Assembly of a Cholera Toxin B Subunit-Rotavirus VP7 Fusion Protein in Transgenic Potato

Nak-Won Choi,[1] Mary K. Estes,[2] and William H. R. Langridge[1,*]

Abstract

A gene encoding VP7, the outer capsid protein of simian rotavirus SA11, was fused to the carboxyl terminus of the cholera toxin B subunit gene. A plant expression vector containing the fusion gene under control of the mannopine synthase P_2 promoter was introduced into *Solanum tuberosum* cells by *Agrobacterium tumefaciens*-mediated transformation. The *CTB*::*VP7* fusion gene was detected in the genomic DNA of transformed potato leaf cells by polymerase chain reaction (PCR) amplification methods. Immunoblot analysis of transformed potato tuber tissue extracts showed that synthesis and assembly of the CTB::VP7 fusion protein into oligomers of pentameric size occurred in the transformed plant cells. The binding of CTB::VP7 fusion protein pentamers to sialo-sugar containing GM1 ganglioside receptors on the intestinal epithelial cell membrane was quantified by enzyme-linked immunosorbent assay (ELISA). The ELISA results showed that the CTB::VP7 fusion protein made up approx 0.01% of the total soluble tuber protein. Synthesis and assembly of CTB::VP7 monomers into biologically active pentamers in transformed potato tubers demonstrates the feasibility of using edible plants as a mucosal vaccine for the production and delivery system for rotavirus capsid protein antigens.

Index Entries: Rotavirus; cholera toxin; edible vaccine; adjuvant; *Solanum tuberosum*.

1. Introduction

Edible plants are a traditional source of food and energy and have been increasingly used throughout the past decade for production and delivery of foreign antigen proteins with therapeutic value. Advances in molecular biology have increased the expression of bacterial and viral antigens in a variety of plant species for use as mucosal vaccines ***(1,2)***. Oral and nasal administrations of plant-produced antigens have elicited mucosal and systemic immune responses in humans and animals, indicating that transgenic plants are feasible production and delivery systems for the development of mucosal vaccines ***(3,4)***. However, because of the degradative environment of the mucosal lining, immunization with plant-produced vaccines is prone to fluctuations in the amount of antigen delivered to immune induction sites (e.g., the gut- or nasal-associated lymphoid tissues [GALT or NALT]) rendering them weakly immunogenic. Thus, enhancement of the immunogenicity of plant-produced antigens would be of significant value.

The heteromultimeric AB_5 cholera toxin isolated from *Vibrio cholerae* has one cytotoxic A subunit (CTA) linked to five identical B (CTB) subunits arranged in a pentameric ring structure ***(5)***. Assembly of five CTB subunit monomers into a pentameric structure is required for binding of approx 7.5×10^4 sugar receptors per mucosal epidermal cell ***(6)***. The cholera toxin B subunit (CTB) has been shown to function as an effective adjuvant molecule for mediating conjugated foreign antigen protein stimulation of mucosal immune

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responses ***(4)*** including delivery of antigens and autoantigens to the GALT ***(7)***. The CTB molecules bind selectively to sialo-sugar-based GM1 ganglioside receptors located on intestinal epithelial cells and M cells mediating antigen uptake ***(8)***. The CTB subunit produced in transgenic food plants has demonstrated its usefulness as an adjuvant for edible vaccine for protection against infectious disease in an animal model ***(9)***. Previous studies indicated that the carboxyl terminus of the CTB monomer is the most effective location for the fusion of foreign antigen proteins for maximum immune stimulation ***(10)***. Thus, CTB has been identified as an adjuvant that stimulates immune responses by targeting fused antigens to the GALT ***(11,12)***.

Rotaviruses are the major worldwide causative agent of acute infantile gastroenteritis. Rotavirus infection is responsible for about 1 million deaths annually in industrialized and developing countries ***(13)*** and an estimated 18 million hospitalizations, of which 20 to 40% are virus-based severe diarrheal illness in infants and young children ***(14)***.

Rotaviruses are nonenveloped, triple-layered icosahedral virus particles containing 11 segments of double-stranded RNA encoding 5 nonstructural proteins and 6 structural proteins ***(15)***. Rotavirus subunit vaccines have increased neutralizing antibodies against VP7, the outer capsid protein that contains the neutralizing epitopes for virus attachment to the host cell ***(16,17)***. The VP7 glycoprotein is responsible for viral serotype determination ***(18)*** and is the outer capsid protein. It is the dominant neutralization antigen in hyperimmune antiserum and constitutes about 30% of the virion protein ***(16)***. The VP7 glycoprotein (38 kDa) has a complex glycosylation pattern identifying 14 serotypes infecting humans ***(19)***. In reassortant rotaviruses, most neutralizing antibodies in hyperimmune antiserum are directed against the VP7 protein ***(20)***. On the basis of amino acid sequence analysis, VP7 has three major antigenic sites in SA11 rotaviruses. They are designated A (amino acids [aa] 87–99), B (aa 145–150) and C (aa 211–223) and are the peptides containing dominant neutralization epitopes ***(21)***. Furthermore, VP7 was shown to be the dominant target protein for rotavirus-specific cytotoxic T-lymphocyte (CTL) activity ***(22)***.

Previous reports showed that plant-produced VP7 antigen generated systemic and mucosal humoral immune responses and generated virus neutralization in orally immunized mice ***(23)***. Alternatively, a VP7 DNA vaccine induced humoral immune responses in mice but was unable to protect mice against virus challenge ***(24)***.

To test the feasibility of generating a strong plant-produced mucosal rotavirus subunit vaccine, we constructed a CTB subunit::rotavirus outer capsid protein VP7 fusion gene and transferred the *CTB*::*VP7* fusion gene into potato plants.

2. Materials and Methods

2.1. Construction of Plant Expression Vector pPCV701CTB::VP7

A *CTB*::*VP7* fusion gene encoding the VP7 amino acid residues 51–326 was constructed using polymerase chain reaction (PCR) cloning methods in *Escherichia coli* (**Fig. 1**). The oligonucleotide 5' primer (5'-GC**GAGCTC**CAAA ATTATGG-3') and the 3' primer (5'-TCC**CC CGGG**CACAC-TCTGTAATAAAATG C-3') were used for amplification of the VP7 gene from VP7 cDNA from simian rotavirus SA11 gene 9. This gene encodes the full-length $VP7_{326}$ protein. The oligonucleotide 5' primer (5'-GC**TC TAGA** GCCACCATGATTAAATT-AAAATT TGGT G-3') and the 3' primer (5'-GC**GAGCT C**CGGG CCTGGGCCATTT- GC-3') were used for amplification of the CTB hinge (CTBh) fusion sequence from plasmid pRT42 containing the *ctxAB* gene from *V. cholerae* strain 569B provided by Dr. John Mekalanos, Harvard Medical School, Boston, MA. The 3' primer for CTB was designed to contain the tetrapeptide hinge (Gly-Pro-Gly-Pro) to allow molecular movement between CTB and the conjugated VP7 peptide ***(25)***. Briefly, the PCR conditions included 30 cycles of PCR amplification (DNA strand denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and complementary strand synthesis at 72°C for 30 s). After completion of the two PCR amplification reactions, the CTB and VP7 gene fragment PCR products were

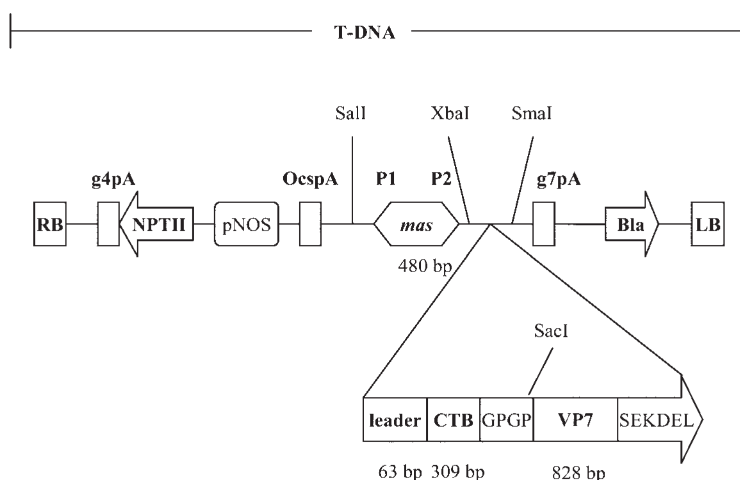

Fig. 1. Plant expression vector pPCV701CTB::VP7. The genes located within the T-DNA sequence flanked by the right and left T-DNA borders (RB and LB) include: the *CTB*::*VP7*::SEKDEL ligand–antigen fusion gene under the control of the *mas* P_2 promoter; an NPT II (neomycin phosphotransferase II) expression cassette for kanamycin selection of transformed plants; and a beta-lactamase gene (Bla) for selection of ampicillin resistance in *E. coli* and carbenicillin resistance in *A. tumefaciens*. The g7pA polyadenylation signal is from gene 7 in the *A. tumefaciens* T_L-DNA; the OcspA polyadenylation signal is from the *A. tumefaciens* octopine synthase gene; the pNOS promoter is from the *A. tumefaciens* nopaline synthase gene; the g4pA polyadenylation signal is from gene 4 in the T_L-DNA. P1 and P2 are the *mas* promoters; GPGP is the hinge region; SEKDEL is the ER retention signal.

digested with *Sac*I restriction endonuclease and ligated at 16°C overnight. A second PCR reaction was initiated to amplify the *CTB*::*VP7* fusion gene fragments. The PCR products were digested with *Xba*I and *Sma*I restriction endonucleases. The amplified fusion gene fragment was ligated into the plant expression vector pPCV701: SEKDEL containing a DNA sequence encoding the endoplasmic reticulum (ER) retention signal (SEKDEL) 3' adjacent to the *mas* P2 promoter ***(26)***. Plasmid DNA in the ligation mixture was transferred into *E. coli* strain HB101 by electroporation ***(9)*** and ampicillin-resistant colonies were isolated after overnight culture at 37°C. To confirm the presence of the correct *CTB*::*VP7* fusion gene, plasmid DNA was isolated from individual colonies of transformants and subjected to DNA sequence analysis in a model 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) with the forward primer (5'-ACCAATACA TTACAC TAGCATCTG-3') specific for the *mas* P2 promoter region and the reverse primer (5'-GACTGA GTGCGATATTATGTGTAATAC-3') specific for the gene 7 poly(A) signal region. The plasmid containing the correct DNA sequence was designated as pPCV701CTB::VP7. This plant expression vector was transferred into *Agrobacterium tumefaciens* strain GV3101 pMP90RK by electroporation before plant transformation experiments ***(9)***.

2.2. Potato Transformation

Potato plants *Solanum tuberosum* cv. Bintje were grown under sterile conditions in Magenta GA-7 culture boxes (Sigma Chemical, Co., St. Louis, MO) on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing 3.0% sucrose and 0.2% Gelrite at 20°C in a light room under cool white fluorescent tube lights (12 μE) set on a 16-h photoperiod regime. Stem explants were excised from the plants with a sterile scalpel and immersed in a culture dish containing an overnight culture suspension of *A. tumefaciens* (1×10^{10} cell/mL) harboring plasmid pPCV701CTB::VP7. The explants were incubated in the bacterial suspension for 15 min, blot-

ted on sterile filter paper, and transferred to MS basal solid medium, pH 5.7, containing 0.4 μg/mL indole-3-acetic acid (IAA) and 2.0 μg/mL benzyl adenine (BA). The stem explants were incubated for 2 d at 20°C on MS solid basal medium containing IAA and BA to permit T-DNA transfer into the plant genome. For selection of transformed plant cells and for counterselection against continued *Agrobacterium* growth, the explants were transferred to MS solid medium containing kanamycin (100 μg/mL) and cefotaxime (300 μg/mL) antibiotics. Transformed plant cells formed calli on the selective medium during continuous incubation for 2 to 3 wk at 20°C in a light room under cool white fluorescent tube lights set at a 16-h photoperiod regime. When the putative transformed calli grew to 5–10 mm in diameter (3–4 wk), the calli were transferred to MS basal solid medium containing 2.0 μg/mL BA and 0.1 μg/mL gibberellic acid (GA_3), 100 μg/mL kanamycin and 300 μg/mL cefotaxime for shoot induction. After 3–6 wk, regenerated shoots were excised from the calli and transferred to MS basal solid medium with antibiotics and without growth regulators to stimulate root formation. After the putative transformed potato plantlets formed roots (3–6 wk), they were transferred to pots in the greenhouse and grown to maturity (2–3 mo).

2.3. Detection of CTB::VP7 *Fusion Gene in Transformed Plants*

Genomic DNA was isolated from potato leaf tissues using a DNeasy Plant mini kit (Qiagen, Valencia, CA). The concentration of genomic DNA was measured by spectrophotometer (at 260 nm). The presence of the *CTB*::*VP7* fusion gene in transformed potato genomic DNA (500 ng) was determined by PCR analysis using the 5' primer of CTB and the 3' primer of VP7 with the same conditions for subcloning as described previously.

2.4. Quantitation of CTB::VP7 Fusion Protein in Transformed Potato Plants

The level of active CTB::VP7 fusion proteins in transformed potato plants and its affinity for GM1 ganglioside receptor binding was evaluated in duplicate samples by quantitative chemiluminescent GM1–enzyme-linked immunosorbent assay (ELISA) methods. The microtiter plate was coated with 100 μL/well of GM1 ganglioside (Sigma) diluted to 3.0 μg/mL in bicarbonate buffer (15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$, pH 9.6), at 4°C overnight. The wells were blocked by adding 300 μL/well of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated at 37°C for 2 h followed by washing three times with PBST (PBS containing 0.05% Tween-20). Known amounts of purified undenatured CTB (Sigma) were added to the wells to generate a standard curve. The wells were loaded with 10-fold serial dilutions (100 μL/well) of a centrifuged plant extract containing total soluble potato tuber proteins in PBS and incubated overnight at 4°C. The plate was washed three times in PBST. The plate was first incubated with 100 μL/well of a primary rabbit anticholera toxin (Sigma) diluted to 1:3000 with 0.5% BSA in PBS at 37°C for 6 h. Antirabbit IgG (Sigma) conjugated with alkaline phosphate diluted to 1:15,000 with 0.5% BSA in PBS was added as a secondary antibody (100 μL/well) and the plates were incubated for 2 h at 37°C. The wells were washed and incubated with Lumi-Phos Plus chemiluminescent substrate (Lumigen, Southfield, MI) at 100 μL/well for 30 min at 37°C. The plate was read in a Micro™ ML3000 Microtiter® Plate Luminometer (Dynatech Laboratories, Chantilly, VA).

2.5. Immunoblot Detection of CTB::VP7 Fusion Protein in Transformed Potato Cells

Transformed potato tuber tissues were analyzed by immunoblot analysis for the presence of *CTB::VP7* fusion gene expression. Tuber tissues first surface-sterilized with a 20% solution of commercial bleach containing—two to three drops of Tween-80 were sliced, and incubated for 7 d on MS basal solid medium containing 5.0 mg/mL 1-naphthaleneacetic acid (NAA) and 6.0 mg/mL 2,4-dichlorophenoxyacetic acid (2,4-D) to activate the *mas* promoters. The auxin-treated tissues were homogenized by grinding in a mortar and pestle at 4°C in extraction buffer (1:1 w/v) (200 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 400 m*M* sucrose, 10 m*M* EDTA, 14 m*M* 2-mercaptoethanol,

1 m*M* phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenate was centrifuged at 17,000*g* in a Beckman GS-15R centrifuge for 15 min at 4°C to remove insoluble cell debris. An aliquot of supernatant containing 100 μg of total soluble protein, as determined by the Bradford protein assay (Bio-Rad, Inc., Hercules, CA), was separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 125 V for 1.5 h in Tris-glycine buffer (25 m*M* Tris-HCl, 250 m*M* glycine, pH 8.3, 0.1% SDS). The separated protein bands were transferred from the gel to approx 80 cm^2-size Immun-Lite membranes (Bio-Rad) by electroblotting on a semidry blotter (Sigma) for 120 min at 30 V and 70 mA. Nonspecific antibody binding was blocked by incubation of the membrane in 25 mL of 5% nonfat dry milk in TBS buffer (20 m*M* Tris-HCl, pH 7.5 and 500 m*M* NaCl) for 1 h with gentle agitation on a rotary shaker (40 rpm), followed by washing in TBS buffer for 5 min. The membrane was incubated overnight at room temperature with gentle agitation in a 1:1000 dilution of rabbit antirotavirus antiserum (provided from Dr. Estes laboratory) in TBST antibody dilution buffer (TBS with 0.05% Tween-20 and 1% nonfat dry milk) followed by washing three times in TBST washing buffer (TBS with 0.05% Tween-20). The membrane was then incubated for 1 h at room temperature with gentle agitation in a 1:10,000 dilution of mouse antirabbit IgG conjugated with alkaline phosphatase (Sigma) in antibody dilution buffer. The membrane was washed three times in TBST buffer as before, followed by incubation in 10 mL of BCIP/NBT alkaline phosphatase substrate (Sigma) for 15 min at room temperature with gentle agitation on a rotary shaker.

3. Results

3.1. Detection of CTB::VP7 Fusion Gene in Transformed Potato Plants

The *CTB*::*VP7* fusion gene was introduced into the T-DNA region of plant expression vector pPCV701, which in turn was integrated into the chromosome of *S. tuberosum* cells by *A. tumefaciens*-mediated in vivo transformation methods. Independently transformed kanamycin-resistant potato explants formed roots after transfer of the transformed shoots to MS basal medium containing the antibiotic kanamycin (100 μg/mL). A DNA fragment (1137 bp) corresponding in size to the *CTB*::*VP7* fusion gene was amplified from the genomic DNA of the transformed potato leaf tissues. No DNA band corresponding to the *CTB*::*VP7* fusion gene was detected in untransformed potato genomic DNA (**Fig. 2**). Transgenic potato genomic DNA extracts were subjected to PCR analysis with primers specific for the plant expression vector region outside the T-DNA region. The results confirmed the absence of contaminating *Agrobacterium* plasmid DNA in the genomic DNA extracts.

3.2. ELISA Quantification of CTB::VP7 Fusion Protein in Transformed Potato Plants

The amount of biologically active oligomeric CTB::VP7 fusion protein produced in undenatured (unboiled) transformed tuber tissue extracts was determined based on the number of relative light units (RLU) generated by chemiluminescent GM1–ELISA methods. Quantification of the number of RLU detected in transformed plant tuber tissues was compared with the RLU generated by a commercial CTB (Sigma) protein standard curve. The amount of recombinant CTB::VP7 fusion protein as a constituent of total soluble plant protein (TSP) was calculated by dividing the amount of CTB-detected sample based on RLU by the TSP identified in the plant tuber tissue extracts as determined by the Bradford protein assay (Bio-Rad). The recombinant protein was found to range from 0.0064 to 0.0082% of TSP or approx 6.4 mg per 100 g of tuber tissue (**Fig. 3**).

3.3. Immunodetection of CTB::VP7 Fusion Protein in Transformed Potato Plants

The CTB::VP7 fusion protein was detected by immunoblot analysis in transformed leaf tissue extracts from putatively transformed plants (data not shown). Microtubers from the transformed potato plants showing high foreign protein expression levels in leaf tissues as measured by ELISA methods were tested by immunoblotting to identify the presence of the CTB::VP7 fusion protein.

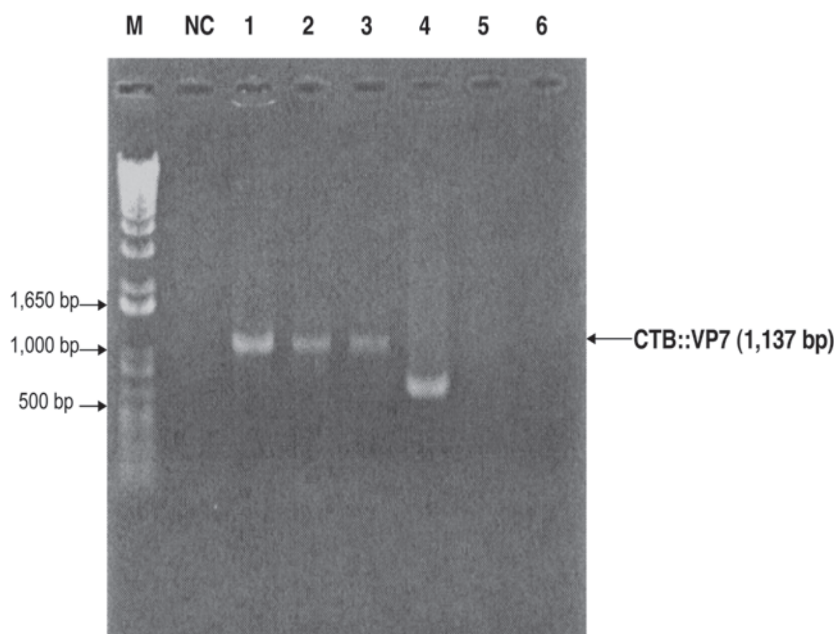

Fig. 2. Detection of the *CTB*::*VP7* fusion gene in transformed potato leaf tissues. Genomic DNA (500 ng) isolated from transformed potato leaf tissues was used to demonstrate the presence of the *CTB*::*VP7* fusion gene in plant chromosomal DNA by PCR analysis with the 59 primer of CTB and the 39 primer of VP7 (used for products of lanes NC, 1, 2, and 3). The PCR amplification with primers (used for products of lanes 4, 5, and 6) specific for the plasmid region excluding the T-DNA (immediately downstream of the T-DNA right border) indicated that no *Agrobacterium* plasmid contamination was present in the genomic DNA extracts. Lane M contains 1 kb plus molecular weight markers (New England Biolabs Inc.); lane NC is untransformed plant genomic DNA used as a negative control; lanes 1 and 4 are pPCV701CTB::VP7 template DNA used as a control for PCR; lanes 2 and 5 are genomic DNA from transformed plant #3; lanes 3 and 6 are genomic DNA from transformed plant #5.

The CTB::VP7 fusion protein was detected in tuber extracts by immunoblot analysis with rotavirus antiserum as the primary antibody (**Fig. 4**). The CTB::VP7 fusion protein monomer (~42 kDa) was detected in boiled extracts of tuber tissue from transformed potato plants. The CTB::VP7 monomers (boiled) assembled into oligomeric structures resembling native pentamers (~210 kDa). The pentamers were detected by immunoblot analysis of transformed potato tuber extracts (**Fig. 4**). No protein band corresponding in size to the CTB::VP7 fusion protein was detected in tuber tissue extracts from untransformed plants.

4. Discussion

Because plant-produced vaccines are relatively inexpensive to produce, safe from pathogenic animal contaminants, and convenient to administer by eating, the application of transgenic plants as vaccine production and delivery vehicles has increased annually for delivery of viral and bacterial pathogen antigens that retain sufficient biological activity and functions for successful mucosal vaccine development ***(27,28)***. Plant cells, similar to other eukaryotic cells, can perform post-translational modifications (e.g., glycosylation and phosphorylation) required for maximum biological activity of proteins of animal origin ***(27)***. In our laboratory, transformed potatoes expressing the CTB subunit genetically linked to enteric viral antigen proteins and autoantigen proteins were found to generate mucosal humoral antibody immune responses that protected mice against rotavirus infection and the autoimmune disease type 1 diabetes ***(4,7)***. These results indicated the

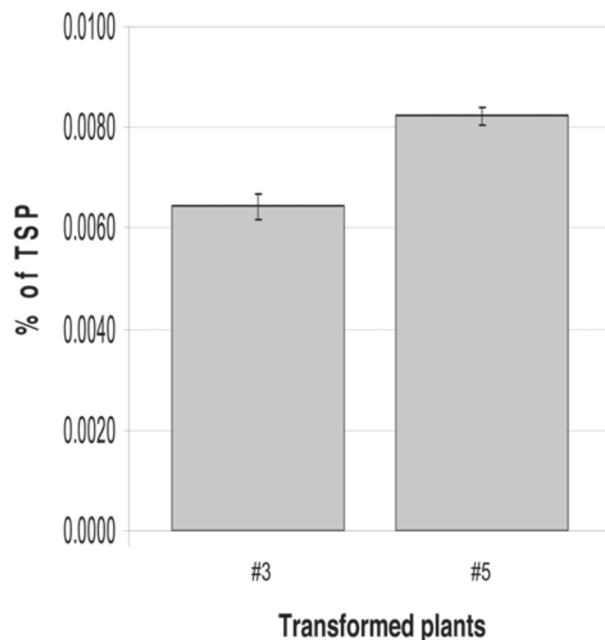

Fig. 3. GM1-ELISA measurement of CTB::VP7 fusion protein levels in transformed plant tuber tissues. Polyclonal anticholera toxin antiserum was used as the primary antibody in the GM1 ganglioside-ELISA. Serial dilutions of total soluble protein from transformed and untransformed plant tuber tissue extracts (unboiled) were measured in duplicate by GM1-ELISA. To calculate the relative light unit (RLU) value for each transformed plant, the untransformed plant RLU value was subtracted from the transformed plant RLU value. The light unit values were compared with commercially prepared cholera toxin B (CTB) subunit standard curve to calculate the CTB::VP7 fusion protein expression levels in the transformed potato plant tuber tissue extracts. The bars represent the percentage of CTB::VP7 fusion protein as part of the total soluble protein (TSP) in the transformed potato tuber tissue extract. Error bars represent the standard deviation from the mean.

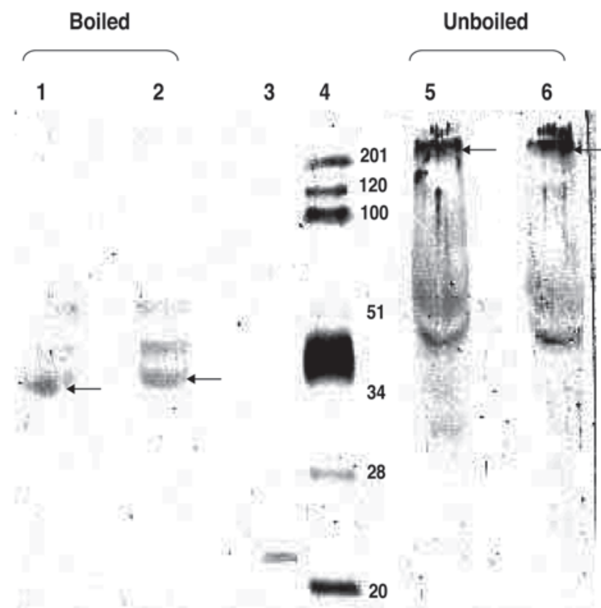

Fig. 4. Immunoblot detection of the CTB::VP7 fusion protein in transformed potato tuber tissues. Extracts from auxin-induced tuber tissues from transformed potato plants were analyzed by immunoblot for expression of the CTB::VP7 fusion protein using antirotavirus antiserum as the primary antibody. Lane 1, CTB::VP7 monomer (~42 kDa) (arrow) from transformed potato plant (#3), tuber tissue extract boiled for 10 min; lane 2, CTB::VP7 monomer (arrow) from transformed potato plant (#5), tuber tissue extract boiled for 10 min; lane 3, tuber tissue extract from untransformed potato plant; lane 4, contains molecular weight markers (Bio-Rad); lane 5, CTB::VP7 pentamer (~210 kDa) (arrow) from tuber tissue extract of unboiled transformed potato plant (#3), (100 μg soluble protein per lane); lane 6, CTB::VP7 pentamer (arrow) of the tuber tissue extract from transformed potato plant (#5), (100 μg soluble protein per lane).

feasibility of generating plant-produced mucosal subunit vaccines for immune protection against both infectious and autoimmune diseases.

Driven by the need for inexpensive oral vaccines for use in both industrialized and developing worlds, current plant-produced edible vaccine research targets predominantly pathogens that cause infectious enteric diseases such as rotavirus, Norwalk virus, cholera, and enterotoxigenic *E. coli* (ETEC), which are the leading causes of epidemic gastroenteritis in humans ***(29)***. The only available reassortant rotavirus vaccine, although effective, was found to cause higher than normal rates of intussusception (lethal infolding of the intestine) in young children ***(30)***. Thus, the development of effective rotavirus subunit vaccines

unlikely to cause intussusception is highly desirable. Although subunit vaccines consisting of individual virus proteins are unlikely to cause intussusception, they may be weakly immunogenic. Thus, a rotavirus subunit mucosal vaccine with the capacity for enhanced immune stimulation would be of value.

In this work, we constructed a *CTB*::*VP7* fusion gene in plant expression vector pPCV701 to test the feasibility of generating mucosal subunit vaccines in potato plants. The rotavirus outer capsid protein VP7 was attached to the CTB carboxyl terminus to facilitate pentamer assembly. The membrane binding the CTB subunit requires assembly of CTB monomers into a pentameric structure possessing the essential geometry for intestinal epithelial cell receptor binding and uptake of ligand-antigen conjugates into the cell for presentation to the GALT for stimulation of humoral and cellular immune responses. Because each CTB monomer has one conjugated VP7 antigen protein in a pentmerized oligomeric structure. One CTB pentamer can carry up to five VP7 antigen proteins. Because CTB monomers are incapable of membrane receptor binding, the performance of CTB carrier functions in animals immunized with the CTB-antigen fusion proteins is dependent on CTB pentamer assembly. Both immunoblot analysis and GM1–ELISA experiments showed that CTB::VP7 fusion proteins assemble into biologically active pentameric structures in transformed tubers. Furthermore, these data indicate that the VP7 protein size does not significantly interfere with CTB fusion protein pentamerization in the plant cell. Genetic conjugation of CTB with virus antigens expressed in transformed potato tubers significantly enhanced the humoral antibody immune responses in comparison with co-administered CTB proteins mixed with antigen, confirming that CTB antigen fusion proteins serve as stronger adjuvants ***(4)***. Earlier studies showed that fusion of CTB to pathogen antigens increases CTB adjuvanticity by more than 10,000-fold ***(31)***. Therefore, it is predicted that the CTB::VP7 fusion protein may generate more effective humoral immune responses for protection against rotavirus infection in comparison to immunization with the VP7 antigen alone. Previous studies in our laboratory and others have shown that inclusion of the CTB signal peptide and the linkage of an ER retention signal (SEKDEL) to the carboxyl terminus of the fusion protein were important for sequestration of the ligand–antigen conjugate in the ER, to facilitate CTB pentamerization and to increase fusion protein levels in transformed plant cells ***(32)***. A flexible hinge peptide (GPGP) was introduced between the CTB and VP7 protein moieties to reduce the possibility of steric hindrance to facilitate CTB receptor binding by allowing maximum intramolecular movement between the CTB and VP7 protein moieties ***(25)***.

The VP7 glycoprotein, located in the virus outer capsid, is the dominant neutralization antigen in hyperimmune antiserum for serotype specificity and constitutes 30% of the virion protein ***(33)***. The importance of glycosylation to the antigenicity of VP7 was shown by the effect of a single amino acid change at amino acid 211, the designated antigenic region C of simian rotavirus SA11 ***(34)***. This region appears to be the dominant antigenic site in SA11 VP7. Alteration of this site renders it inaccessible to neutralizing antibodies by addition of a new carbohydrate side chain. Although various structural and nonstructural rotavirus proteins have been reported to elicit a CTL response in mice, the VP7 protein is a major target of rotavirus-specific CTLs. Previous reports showed that antiserum raised against baculovirus-expressed VP7 neutralized SA11 rotavirus infectivity ***(24)***, whereas recombinant VP7 expressed in *E. coli* proved to be toxic to the bacteria ***(35)***. Furthermore, the premature form of membrane bound VP7 found in the ER did not induce neutralizing antibody because its conformation was different from the mature form of VP7 found in the assembled virus ***(36)***.

Based on the ELISA results, expression levels of 6.4 mg of CTB::VP7 fusion protein per 100 g of transformed potato tuber tissues will provide approx 19.2 mg of CTB::VP7 fusion protein in an average 300 g of transformed potato tuber. On the basis of the standard oral dose of 1.0 mg per dose of CTB delivered to humans for protection against cholera toxin ***(37–39)***, a single potato could pro-

vide several protective doses. The amount of biologically active CTB::VP7 fusion protein in total soluble plant protein was calculated based on the results of GM1–ELISA binding experiments in which only pentameric CTB is able to bind to the microtiter plate. Although unbound monomeric CTB::VP7 would not be included in this calculation, it could be expected to participate in induction of the immune response after mucosal delivery and uptake by dendritic cells, macrophages, and B cells, and the resultant presentation of immunogenic CTB::VP7 peptides to Th lymphocytes in the GALT.

In a previous report rotavirus VP7 antigen produced in transformed potato induced secretory IgA in the intestinal contents of immunized mice as a first line of defense against virus infection ***(23)***. Furthermore, it has been suggested that CTB may have an adjuvant effect in mucosal subunit vaccines composed of plant-produced rotavirus antigens ***(4)***.

In this study we have generated transformed potato plants capable of synthesizing detectable amounts of CTB subunit::rotavirus outer capsid VP7 fusion protein. The ability of plant-synthesized CTB::VP7 fusion protein to generate protective humoral and cellular immune responses against rotavirus challenge will be the subject of analysis in the next animal immunization experiments.

Acknowledgments

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