Cholera Toxin B Subunit-*Porphyromonas gingivalis* Fimbrial Antigen Fusion Protein Production in Transgenic Potato

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Abstract *Porphyromonas gingivalis*, the gram-negative anaerobic oral bacterium, initiates periodontal disease by binding to saliva-coated oral surface. The cholera toxin B subunit (CTB) genetically linked to FimA1 (1-200 aa) or FimA2 (201-337 aa) of the *P. gingivalis* fimbrial antigen were introduced into *Solanum tuberosum* cells by *Agrobacterium tumefaciens*-mediated transformation method. The integration of CTB-FimA1 or CTB-FimA2 fusion genes were confirmed in the chromosome of transformed leaves by genomic DNA PCR amplification method. Synthesis and assembly of the CTB-FimA fusion proteins into oligomeric structures with pentamer size was detected in transformed tuber extracts by immunoblot analysis. The binding activities of CTB-FimA fusion proteins to intestinal epithelial cell membrane receptors were confirmed by G_{M1}-ganglioside enzyme-linked immunosorbent assay (G_{M1}-ELISA). The ELISA showed that the expression levels of the CTB-FimA1 or CTB-FimA2 fusion proteins were 0.0019, 0.002% of the total soluble protein in transgenic tuber tissues, respectively. The synthesis of CTB-FimA monomers and their assembly into biologically active oligomers in transformed potato tuber tissues demonstrates the feasibility of using edible plants for the production of enterocyte targeted fimbrial antigens that could elicit mucosal immune responses.

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Introduction

Transgenic plants and plant cell suspension cultures have been used for production of pharmaceutically or industrially useful recombinant proteins; including enzymes, vaccines, hormones, and antibodies (McGarvey et al. 1995; Arakawa et al. 1999; Kim et al. 2004; Kim and Langridge 2004). Transgenic plants have several advantages compared to animal and bacterial expression systems; low price of medium, safe from contamination of animal pathogens, easy to store and transport, and without the risk of needle-associated injury and disease spread. Plant-based vaccines are very attractive in developing countries because many peoples were not vaccinated due to financial problems and suffered from diseases. However, the low expression level of vaccine antigens in transgenic plants elicited the low immune responses and is a potential drawback for using of plant-based vaccine to protect host from diseases. In order to overcome this obstacle, many scientists tried to increase the expression level of target antigen genes in transgenic plants by the strong promoters, codon optimization (Kang et al. 2004), chloroplast transformation (Kang et al. 2003). It is alternative way to increase the uptake of vaccine antigens into immune systems by fused with bacterial toxin B subunit which has an ability to enter mucosal immune tissue together with fused proteins when orally administrated (Kim et al. 2004).

Bacterial toxins consist of A and B domains, and B domains have been used to increase antigens uptake into gut epidermal cells for increasing of the mucosal immune responses

(Arakawa et al. 2001; Kim et al. 2004). Cholera toxin (CT) is a typical representative of the heteromultimeric AB toxins produced by *Vibrio cholera* and one of the most effective enterocyte targeting molecules. The CT molecule consists of one A subunit and five B subunits. The CTB subunit is composed of five identical polypeptides (11.5 kDa) that assemble into a highly stable pentameric ring structure in the bacteria. The CTB pentamer binds selectively to sugar-lipid G_{M1}-ganglioside molecules embedded in the membranes of intestinal epithelial (enterocytes) and microfold (M) cells (Dertzbaugh and Elson 1993). The CTB has been shown to function as an effective carrier molecule for fused foreign proteins including mucosal vaccine antigens and autoantigens (Sun et al. 1994; Weiner 1994).

One of oral pathogens, *Porphyromonas gingivalis*, is a black-pigmented gram-negative anaerobe, and has been implicated in the pathogenesis of severe periodontal disease in adults (Lamont and Jenkinson 1998; Hamada et al. 1998). In the first infection step of the oral cavity, bacterial adherence to the oral mucosal surface is important. The binding of *P. gingivalis* to the oral surface is mediated by fimbrial interactions with salivary proteins (Lee et al. 1992; Amano et al. 1996), fimbronetin (Soja et al. 1995), and cytokeratins (Soja et al. 2002). Fimbriae are of particular candidates for the development of vaccine because they are one of the critical cell surface virulence factors of *P. gingivalis*. Patients with destructive periodontal disease have markedly elevated serum and gingival fluid antibody responses to *P. gingivalis* fimbriae (De Nardin et al. 1991; Condorelli et al. 1998). Antibodies raised against the fimbriae provide protection against periodontal disease. Monoclonal antibodies to fimbriae completely inhibit *P. gingivalis* binding (Isogai et al. 1988). In rabbits, *P. gingivalis* fimbriae induce opsonic antibodies (Fan et al. 2001). Moreover, inactivation of FimA gene encoding fimbrillin, a structural subunit of fimbriae, results in a decrease in the ability of *P. gingivalis* to interact with salivary components adsorbed to hydroxyapatite beads (Malek et al. 1994), and the invasion of epithelial cells (Weinberg et al. 1997; Umemoto and Hamada 2003). These results suggest that fimbriae play a crucial role in the bacterial interactions with host tissues and in the pathogenesis of periodontal disease and possibly linked to inflammation-related disease such as cardiovascular disease.

In this work, FimA1 (1-200 aa) and FimA2 (201-337 aa) genetically linked to cholera toxin B subunit fusion genes were constructed and introduced into potato to test the feasibility of expression and assembly of CTB-FimA proteins in the tuber tissue of transgenic potatoes as carrier and adjuvant to increase the protective efficacy of mucosal vaccination against *P. gingivalis* infections.
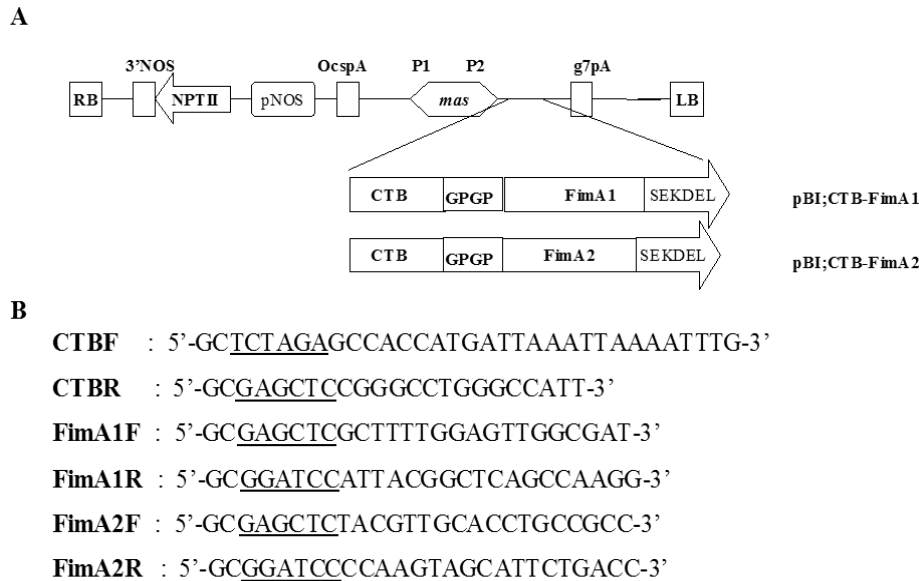
Materials and methods

Construction of plant expression vectors

The FimA1, FimA2 and CTB genes were amplified from plasmid pET-11d containing the FimA gene (Nagata et al. 1997) and plasmid pPCV701;CTB-NSP4 (Kim and Langridge 2003) with gene specific primers (Fig. 1). The amplification program proceeded as follows; 10 min of denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final, 10-min extension step at 72°C. The PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI). The correct DNA sequence of PCR products was confirmed by DNA sequence analysis. After confirmation of the DNA sequences, the FimA1 or FimA2 were fused with the 3' end of CTB gene. The recombinant CTB-FimA fusion genes were introduced into plant binary vector under the control of mas P2 promoter (Velten et al. 1984). The recombinant plasmids were designed pBI;CTB-FimA1 and pBI;CTB-FimA2, respectively. The recombinant plasmids were transformed into *Agrobacterium tumefaciens* LBA4404 by Tri-parental mating method (Van haute et al. 1983).

Potato transformation

Stem explants of potato plants (*Solanum tuberosum* cv. Bintje) grown under sterile conditions were transformed with *A. tumefaciens* harboring pBI;CTB-FimA1 or pBI;CTB-FimA2. Briefly, stem explants were cut with 0.5 cm length and incubated in the *Agrobacterum* suspension culture for 15 min. The explants were blotted on sterile filter paper and transferred to MS basal solid medium (Murashige and Skoog 1962), pH 5.7, containing plant growth regulators, 0.4 μg/mL indole-3-acetic acid (IAA) and 2.0 μg/mL benzyl adenine (BA), and incubated under dark condition for 2 d at 20°C. For selection, the explants were transferred to MS solid medium containing kanamycin (100 μg/mL) and cefotaxime (300 μg/mL). Transformed plant cells formed calli on the selective medium after 2 to 3 weeks incubation at 20°C in a light room on a 18 h photoperiod. Putative transformed calli were transferred to MS basal solid medium containing 2.0 μg/mL BA and 0.1 μg/mL gibberellic acid (GA3), 100 μg/mL kanamycin and 300 μg/mL cefotaxime for shoot induction. After 3-6 weeks further incubation in the light room, regenerated shoots were excised from the calli and transferred to MS basal solid medium with antibiotics and without growth regulators to stimulate root formation. The putative transformed potato plantlets formed roots in 3-6 weeks.

A

B

CTBF   : 5'-GC<u>TCTAGA</u>GCCACCATGATTAAATTAAAATTTG-3'

CTBR   : 5'-GC<u>GAGCT</u>CCGGGCCTGGGCCATT-3'

FimA1F : 5'-GC<u>GAGCT</u>CGCTTTTGGAGTTGGCGAT-3'

FimA1R : 5'-GC<u>GGATCC</u>ATTACGGCTCAGCCAAGG-3'

FimA2F : 5'-GC<u>GAGCT</u>CTACGTTGCACCTGCCGCC-3'

FimA2R : 5'-GC<u>GGATCC</u>CCAAGTAGCATTCTGACC-3'

Fig. 1 Structure of plant expression vector for CTB-FimA fusion genes. Genes located within the T-DNA sequence flanked by the right and left borders (RB and LB) include the CTB-FimA1 or CTB-FimA2 fusion genes under the control of mas P2 promoter. The neomycin phosphotransferase II gene (NPTII) is under the control of nopaline synthase promoter (pNOS) and terminator (3'NOS) as a selection marker. The OcspA and g7pA are polyadenylation signals from the octopine synthase gene and gene 7 in TL-DNA of *A. tumefaciens*, respectively. GPGP denotes a flexible hinge tetrapeptide between CTB and FimA, and SEKDEL indicates the presence of an ER retention signal (A). The primers for amplification of CTB, FimA1 and FimA2 were listed in (B). Underlines represent restriction enzyme sites used for gene construction

Detection of CTB-FimA genes in transformed potatoes

Genomic DNA was isolated from putative transformed potato leaf tissues using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The concentration of genomic DNA was measured in a UV spectrophotometer (at 260 nm). The presence of the CTB-FimA1 or CTB-FimA2 fusion genes in transformed potato genomic DNA (400 ng) was determined by PCR analysis with the same conditions for subcloning as described above.

Detection of the CTB-FimA fusion proteins in transformed potatoes

The tuber tissues of transformed potato were analyzed by immunoblot analysis for production of the CTB-FimA fusion proteins. The transgenic tubers were sliced and incubated for 5 d on MS basal solid medium containing 5.0 mg/L 1-naphthaleneacetic acid (NAA) and 6.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) to activate the mas promoters. The tissues were homogenized by grinding in a mortar and pestle at 4°C in extraction buffer (1:1 w/v) (200 mM Tris-Cl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenate was centrifuged at 17,000 x g in a Beckman GS-15R centrifuge for 15 min at 4°C to remove insoluble cell debris. An aliquot of the su-

pernatant containing 100 μg of total soluble protein, as determined by the Bradford protein assay (Bio-Rad, Inc., Hercules, CA), was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1.5 to 2 h in Tris-glycine buffer (25 mM Tris-Cl, 250 mM glycine, pH 8.3, 0.1% SDS). Prior to electrophoresis, unboiled and boiled samples were loaded on the gel. Boiling the sample for 5 min ensured protein denaturation. Following electrophoresis, the separated protein bands were transferred from the gel to nitrocellulose filters. Nonspecific antibody binding was blocked by incubation with 5% non-fat dry milk in TBS buffer (20 mM Tris-Cl, pH 7.5 and 500 mM 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 1:2,000 dilution of rabbit anti-cholera toxin antibody (Sigma C-3062, St. Louis, MO) followed by three washes in TBST washing buffer (TBS with 0.05% Tween-20). The membrane was incubated for 1 h at room temperature with gentle agitation in a 1:7,000 dilution of mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-2556). The membrane was washed three times in TBST buffer as before, and incubated in 10 ml of BCIP/NBT alkaline phosphatase substrate (Sigma B-5655) for 15 min at room temperature with gentle agitation on a rotary shaker to develop an image of the protein bands.

Quantitation of CTB-FimA fusion proteins in transformed potatoes

The expression levels of CTB-FimA fusion protein in transformed potato tuber tissues were quantified by G_{M1}-ELISA methods. Briefly, the microtiter plate was coated with 100 μL per well of monosialoganglioside G_{M1} (3.0 μg/mL) (Sigma G-7641) dissolved in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$), pH 9.6, covered with Saran wrap and incubated at 4°C overnight. The wells were blocked by adding 200 μL/well of 1% bovine serum albumin (BSA) in PBS and incubated at 37°C for 2 h followed by washing three times with PBST (PBS containing 0.05% Tween-20). The wells were loaded with serial dilutions (100 μL per well) of protein extracts in PBS and incubated overnight at 4°C. The wells were washed three times with PBST and loaded with 100 μL per well of a 1:8,000 dilution of rabbit anti-cholera toxin primary antibody and incubated for 2 h 37°C, followed by washing the wells three times with PBST. The plate was then incubated with 100 μL per well of secondary antibody, a 1: 20,000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma A-2556) for 2 h at 37°C and washed three times with 300 μL per well of PBST. Plates were finally incubated for 30 min at room temperature with 100 μL per well of TMB substrates L (PharMingen 2606 and 2607KC, Fallbrook, CA). Optical density was measured at 405 nm wavelength in an ELISA reader (Packard Instrument MRA-006, Meriden, CT).

Results

Detection of CTB-FimA fusion genes in transformed potato plants

The CTB-FimA1 (1-200 aa) and CTB-FimA2 (201-337 aa) genes were inserted into plant expression vector by PCR cloning methods (Fig.1) to produce the respective CTB-FimA fusion proteins in transgenic potatoes. Independently transformed kanamycin-resistant potatoes formed roots 3-5 weeks following transfer of transformed shoots to MS basal medium containing antibiotic kanamycin (100 μg/mL). The integration of target genes into the chromosome of transgenic plants was confirmed by PCR amplification method. PCR products corresponding to the CTB-FimA1 (1,000 bp) were detected in 2 out of 3 transformed potato plants with pBI;CTB-FimA1 and no DNA band corresponding to the CTB-FimA1 fusion gene was detected in untransformed potato genomic DNA. DNA fragments corresponding to the CTB-FimA2 (800 bp) fusion genes were amplified from the genomic DNA of three
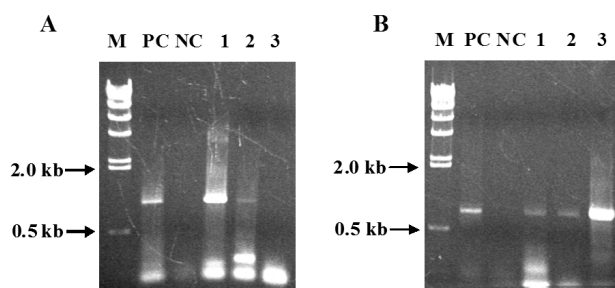
Fig. 2 Genomic DNA PCR analysis in transgenic plants. PCR analysis of transgenic and wild type plants was conducted to amplify the CTB-FimA1 (A) or CTB-FimA2 fusion genes (B). Lane M is lamda/HindIII DNA size marker. Lane PC is plant expression vector used as a positive control for PCR; lane NC is wild type plant used as a negative control; lanes 1-3 are PCR products amplified from the DNA templates of independent transgenic lines

transformed potato leaf tissues with pBI;CTB-FimA2 and PCR products corresponding to the CTB-FimA2 fusion gene was not detected in untransformed potato (Fig. 2).

Detection of plant-produced CTB-FimA fusion proteins

The CTB-FimA1 (~34 kDa) or CTB-FimA2 (~27 kDa) fusion proteins were detected by immunoblotting of putatively transformed potato microtuber tissue extracts with anti-CTB antibody. The microtubers of two selected transformed potatoes with CTB-FimA1 and three selected transformed potatoes with CTB-FimA2 were ground and extracted with protein extraction buffers after induction with auxin phytoregulators. The presence of CTB-FimA1 or CTB-FimA2 fusion proteins were detected in unboiled tuber tissue extracts by immunoblot analysis with anti-cholera toxin antibody as the primary antibody (Fig. 3). No signal was detected in boiled tuber tissue extracts from CTB-FimA1 plants and boiled or unboiled tuber tissue extracts from untransformed plants. Assembly of CTB-FimA1 or CTB-FimA2 fusion proteins into oligomeric structures was detected on immunoblots of unboiled transformed potato tuber extracts (Fig.3).

ELISA quantification of CTB-FimA fusion proteins in transformed potato plants

The amount of CTB-FimA fusion proteins synthesized in transformed microtuber tissues was measured by G_{M1}-ELISA methods. The amount of CTB-FimA recombinant proteins as a constituent of total soluble tuber protein (TSP) was calculated by dividing the amount of CTB-FimA proteins detected based on optical density (OD) by the TSP identified in the plant tissue as determined by the Bradford
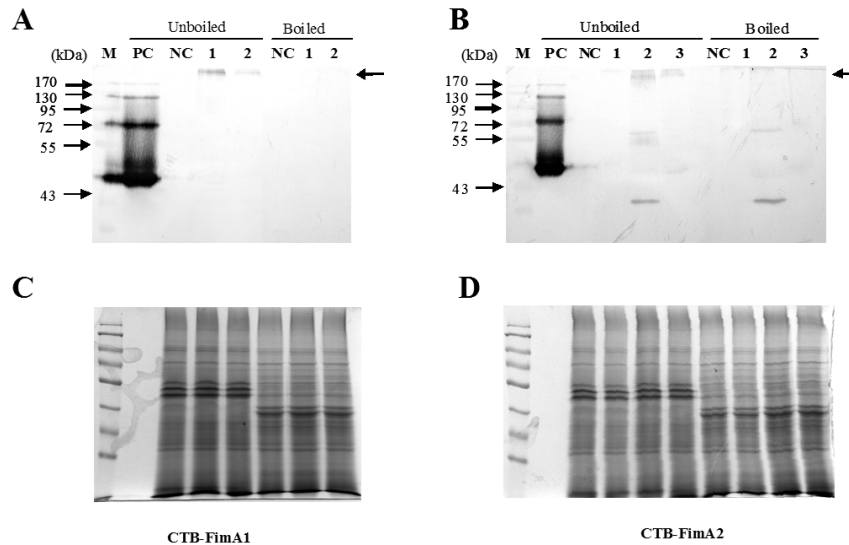
Fig. 3 SDS-PAGE and Western blot analysis of CTB-FimA fusion proteins expressed in the tuber tissues of transgenic potato plants. Total soluble protein extracts (15 μg) from the tuber tissues of wild type (NC) and transgenic plants along with 80 ng of purified bacterial CTB protein were separated on SDS-PAGE. Denatured proteins were boiled for 5 min prior to loading on the gel. Panel A and C show Western blot analysis and SDS-PAGE of CTB-FimA1 fusion protein, and panel B and D show Western blot analysis and SDS-PAGE of CTB-FimA2 protein expressed in transgenic tubers. Lane M is Prestained Protein Ladder (Fermentas, Glen Burnie, MD). Lane PC is bacterial CTB. Lanes 1-3 are transgenic tuber protein extracts
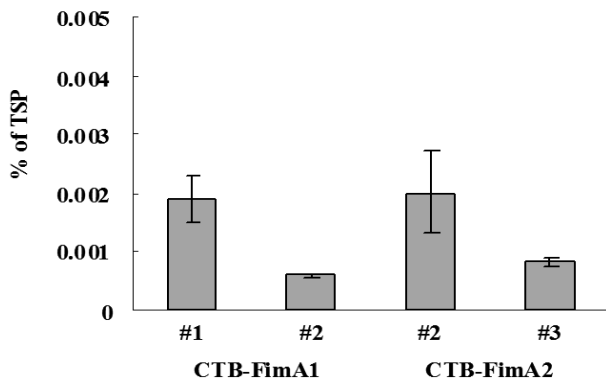
Fig. 4 The quantification and biological activity of CTB-FimA fusion proteins produced in transgenic plants. The CTB-fusion proteins expression level (% of TSP) in the tuber tissues of transgenic potato plants using G_{M1}-ELISA. The G_{M1}-ELISA was conducted in triplicates with coating the plates with G_{M1}-ganglioside as receptor molecules which is a receptor for biologically active CTB. Error bars represent standard deviation of the mean.

protein assay of tissue extracts. The amounts of recombinant protein in transformed tuber tissue with CTB-FimA1 or CTB-FimA2 were found to be 0.0019 or 0.002% of TSP, respectively (Fig. 4).

Discussion

P. gingivalis is one of a fine finite number of anaerobic bacterial species significantly associated with severe periodontal disease (Loesche and Grossman 2001). The fimbriae are one of the critical cell surface virulence factors in *P. gingivalis* to interact with endothelial cells and are interest to the development of vaccine for protection against periodontal diseases. We previously constructed the transgenic potatoes expressing fusion proteins consisted of cholera toxin B subunit and *P. gingivalis* fimbrial antigen (266-337) in an effort to increase uptake of fused antigens into the enterocyte for improvement of immune responses (Shin et al. 2006). The immune responses from mice oral immunized with bacterial CTB-NSP4$_{90}$ protein showed the higher titers than that in mice immunized with CTB-NSP4$_{22}$, indicating that the larger antigens could elicit more immune responses because larger antigens can contain more epitopes (Kim et al. 2004). In this study, the full-length fimbrial antigens were divided into two parts, FimA1 (1-200 aa) and FimA2 (201-337 aa), to reduce the steric hindrance for pentamer formation of CTB-fusion proteins (Liljeqvist et al 1997; Dertzbaugh and Elson 1993). Assembly of CTB fusion proteins into pentameric structure is considered to be essential for CTB binding to G_{M1}-ganglioside receptors on the enterocyte membrane and uptake of ligand-antigen conjugates into the cell for presentation to lymphoid tissues of GALT for immune stimulation (Dertzbaugh and Elson 1993). Both immunoblot and G_{M1}-ELISA analysis experiments showed that recombinant CTB-FimA1 and CTB-FimA2 assemble into biologically active oligomeric structures in transformed tuber tissues. Previous studies indicated that the CTB signal peptide and the ER retention signal linked to the carboxyl terminus of the fusion proteins

were important for sequestration of the CTB-antigen conjugate into the ER, facilitating CTB-antigen fusion assembly and increased antigen fusion protein gene expression levels in plants (Haq et al. 1995).

Since periodontal care is unable to restore once-damaged periodontal tissue to its original condition, damaged periodontal tissue remains continuously vulnerable to bacterial infection, and periodontal disease often recurs at a later time. Therefore, institution of a global periodontal health system should be oriented toward prevention. Preventive vaccination has been the most cost-effective medical intervention methods available for control and eradication of infectious diseases and their epidermics worldwide. In 1990, the World Health Organization (WHO), launched the Children's Vaccine Initiative establishing goals for the development of mucosal vaccines that are safe, inexpensive, easily (orally) administered, widely accessible for distribution and capable of being stored without refrigeration (Mitchell et al. 1993). Establishment of these goals, the advent of the modern methods of molecular genetics, and molecular biology have ultimately led to development of the biotechnology of oral vaccine production in transgenic food plants.

Transgenic potatoes have been used for production of vaccine antigens including LTB, CTB, and CTB- and LTB-fusion antigens because transformation of potato is easier than other plants and the expression level of target proteins showed relatively higher than other plants. In this work, the amount of CTB-FimA fusion proteins in transgenic potato was low (0.0019 or 0.002% of TSP). The low expression level of vaccine antigens in transgenic plants elicited the low immune responses and is a potential drawback for using of plant-based vaccine. Improvement of expression level of CTB-fimA fusion proteins in transgenic tuber by strong promoters, modification of gene based on plant-optimized codon could be useful to induce efficacious immune responses to protect host against diseases.

In this study, transgenic plants were developed to express the cholera toxin B subunit-*P. gingivalis* fimbrial antigen fusion proteins. The immunogenicity of plant-produced CTB-FimA fusion proteins will be tested in future.

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