

# Synthesis and assembly of dengue virus envelope protein fused to cholera toxin B subunit into biologically active oligomers in transgenic tomato (*Solanum lycopersicum*)

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**Abstract** Dengue is the fastest growing mosquito-borne disease worldwide, causing nearly 400 million infections annually. A universally applicable dengue virus vaccine is required to arrest its spread. Here, we generated an edible dengue vaccine by expressing the dengue fusion protein in tomatoes, which is a desirable expression system owing to the inherent adjuvanticity of alpha tomatine and immunogenicity of the tomato lectin/microbial antigen complex. The B subunit of *Vibrio cholera* toxin (CTB) was genetically fused to dengue envelope antigen for improved delivery to antigen-presenting cells and enhanced immunogenicity, while avoiding immunological tolerance. We utilized domain III of the dengue envelope protein (EDIII), as it has been shown to induce serotype-specific neutralizing antibodies. The CTB–EDIII fusion gene construct containing an endoplasmic reticulum target sequence was introduced into tomato plants by *Agrobacterium tumefaciens*-mediated gene transformation, and the expression of CTB–EDIII in transgenic plants was confirmed by DNA, RNA and protein analyses. Accumulated fusion protein accounted for up to 0.015 % of total soluble protein, and it assembled into fully functional pentamers as demonstrated by binding to GM1 ganglioside. Future work will involve testing of transgenic tomatoes for immunogenicity in mice following oral delivery.

**Keywords** Dengue · Plant-based vaccine · Transgenic tomato · Cholera toxin B subunit

## Introduction

Dengue is one the most common mosquitoes-borne human infectious diseases worldwide. It is caused by dengue virus belonging to the Flavivirus group, which includes viruses such as those causing yellow fever, West Nile, Zika and Japanese encephalitis. The dengue epidemic has increased rapidly since the 1950s and has emerged as a global problem with a high economic burden. One third of the world's population lives in dengue-endemic areas, which span 100 countries within Asia, the Americas, the Western Pacific, Africa and Eastern Mediterranean regions. As many as 400 million people are infected annually, and the burden of dengue infection is likely higher than that reported by national surveillance systems due to unreported cases in developing countries. Dengue virus is a leading cause of illness and death, especially in children under 15 years of age (Gurugama et al. 2010; Sirivichayakul et al. 2012). To lower this risk substantially, an efficacious vaccine is urgently needed; however, a universal dengue vaccine is not yet available. There are four dengue virus serotypes, which have 60–75 % amino acid (aa) sequence homology. However, each of the four serotypes can cause dengue infection in an antigenically distinct manner, which can induce severe symptoms such as fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Antibody-dependent enhancement (ADE) of infection can occur in vivo (Balsitis et al. 2010; Moi et al. 2013) due to increased viral uptake by cells mediated by non-neutralizing antibodies (Huang et al. 2016). Paradoxically, a protective immune response against one or two dengue virus serotypes could actually increase

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the risk of potentially fatal illness through ADE. Therefore, a successful dengue vaccine must be capable of simultaneously inducing high-level long-lasting immunity against all four serotypes.

Dengue virus consists of three structural proteins [capsid, membrane and envelope (E) proteins] and seven non-structural proteins (NS) (Guzman et al. 2010a). The E protein or domain III of E protein (EDIII, 299–393 aa) has been studied extensively as a subunit vaccine candidate (Guzman et al. 2010b; Ketloy et al. 2016; Wahala et al. 2012). NS proteins induce type-specific protective immunity and a majority of the CD4 and CD8 T cell responses (Halstead 2013). The recently developed tetravalent vaccine Dengvaxia, produced by Sanofi Pasteur, is based on live attenuated virus (McArthur and Edelman 2015; Sabchareon et al. 2012; Züst et al. 2013). Another live attenuated vaccine candidate, TV003, developed by the National Institute of Allergy and Infectious Diseases (NIAID) in the USA, was fully protective against dengue in a human challenge model (Kirkpatrick et al. 2016) and is currently undergoing a phase 3 clinical trial in Brazil. Other vaccine candidates based on DNA (Ketloy et al. 2016) and protein (Chiang et al. 2014) are also under consideration as viable alternatives to the attenuated virus-based vaccines.

Plant-based vaccines are an attractive possibility, owing to their superior safety profile and low production cost. Such vaccines have been explored for the past three decades, although none has yet been licensed. However, plant-based oral vaccines are of particular interest due to their potential for protecting the protein antigen in a gastric acid environment. The encapsulated antigens expressed in plant cells have been shown to be an efficient mode of antigen delivery to immune cells by oral administration in mice (Kwon et al. 2013; Limaye et al. 2006). In an experiment to induce tolerance, antibody formulated coagulation factor VIII, cholera toxin B (CTB)-heavy chain and CTB-C2 administered orally to gut-associated lymphoid tissue (GALT) demonstrated binding to dendritic cells in the lamina propria and Peyer's patches of the small intestine (Su et al. 2010). A recent review by Daniell et al. listed many examples of plant-derived oral vaccines against human pathogens, including human papillomaviruses (HPVs), influenza virus, tuberculosis, hepatitis B virus (HBV), human immunodeficiency virus (HIV), rabies and malaria (Chan and Daniell 2015). Antigens from such human infectious agents have been introduced into plant cells subsequently tested as potential oral vaccines. In particular, tomatoes show great oral vaccine potential, as they contain tomato lectin, a known vehicle for gut antigen delivery, and alpha tomatine, a powerful plant endogenous adjuvant (Carreno-Gomez et al. 1999; Morrow et al. 2004). Therefore, we designed a tomato-derived dengue vaccine candidate and investigated its feasibility as a potential oral vaccine candidate.

## Materials and methods

### Transformation and regeneration of plants

Previously, we generated a DNA construct encoding cholera toxin B subunit fused to dengue virus E glycoprotein domain III (CTB–EDIII) with an endoplasmic reticulum (ER) retention signal peptide (Kim et al. 2010b, 2013). To develop a plant-based dengue vaccine, the plant expression vector containing the CTB–EDIII construct was transformed into tomatoes (*Solanum lycopersicum*) using the *Agrobacterium*-mediated gene transformation method. For tomato transformation, commercial tomato seeds were surface-sterilized using 70 % ethanol for 1 min and 10 % sodium hypochlorite with several drops of Tween 20 for 10 min, and then germinated on Murashige and Skoog (MS) basal medium supplemented with 3 % sucrose and 0.8 % plant agar (pH 5.7) at 25 °C in the dark for 5–6 days. The cotyledons were collected from the germinated plants under aseptic conditions, cut on both sides and incubated with *Agrobacterium* suspension solution for 15 min to allow penetration into the plant cells. To remove the remaining *Agrobacterium* from the surface, explants were washed twice with sterilized H<sub>2</sub>O and blotted with filter paper. The *Agrobacterium*-penetrated explants were co-cultured on MS solid medium in the dark for 2 days. The transformed explants were selected on MS medium containing antibiotics plus plant growth hormones (MS basic medium + 1 mg/L zeatin + 0.02 mg/L indole-3-acetic acid (IAA) + 100 mg/mL kanamycin + 300 mg/mL cefotaxime) and cultivated for 4–5 weeks. To obtain the regenerated plants with roots, the shoots induced from transformed explants were transferred to hormone-free MS solid medium. The plantlets with good developed roots and 7–10 cm in size were transferred to soil containing vermiculite and peatmoss and grown up in greenhouse (at 25 ± 2 °C under an intensity of 2000–3000 lx and with a photoperiod of 10 h).

### PCR amplification of CTB–EDIII gene in transformed plants

Genomic DNA was isolated from wild-type and putative transgenic tomato leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) to confirm integration of the target gene into the plant genome. The CTB–EDIII fusion gene was amplified using CTB–EDIII-specific primers (forward primer: 5'-GGT CTA GAG GAT CCG CCA CCA TGG TGA AG-3' and reverse primer: 5'-GCG GTA CCT TTC TTG AAC CAG TTG AG-3') by polymerase chain reaction (PCR) assay.

### Northern blot analysis to detect CTB–EDIII transcripts

To confirm the expression of mRNA, total RNA was extracted from wild-type and transgenic tomato plants using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA samples (30 µg) were concentrated by ethanol precipitation, fractionated by formaldehyde–agarose gel electrophoresis and transferred onto Hybond N<sup>+</sup> membrane (GE Healthcare Life Science Biotech, UK). The membrane was hybridized with a <sup>32</sup>P-labeled CTB–EDIII probe using the Prime-a-Gene labeling system (Promega, Madison, WI, USA) at 65 °C in a hybridization incubator (FINEPCR Combi-H, Seoul, Korea). To visualize the transcripts of CTB–EDIII, the blotted membrane was exposed to X-ray film (Kodak, Rochester, NY, USA) after washing twice with 2× SSC (300 mM NaCl, 30 mM Na-citrate) buffer containing 0.1 and 1 % sodium dodecyl sulfate (SDS) for 15 min at 65 °C.

### Immunoblot analysis of CTB–EDIII protein

To verify the expression of fusion protein in transgenic plants, total soluble protein (TSP) was extracted in protein extraction buffer (200 mM Tris–HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 0.05 % Tween 20). The extracted TSP was quantified by the Bradford protein assay (Bio-Rad, Hercules, CA, USA), and 50 µg was electrophoresed on 8 and 12 % polyacrylamide gels containing SDS, under non-reducing (NR) and reducing (R) conditions, respectively. The purified recombinant CTB or dengue EDIII proteins alone, and wild-type plant extract were used as controls for blotting. The separated protein bands were blotted onto Hybond C membranes (Promega) in transfer buffer (50 mM Tris, 40 mM glycine, and 20 % methanol) using a mini-transblot apparatus (Bio-Rad) at 130 mA for 2 h and detected with anti-CT antiserum (1:5000; Immunology Consultants Lab, Newberg, OR, USA) or anti-dengue monoclonal antibodies (1:2500; AbD Serotech, Oxford, UK). The proteins on the membrane were detected by alkaline phosphatase conjugated anti-rabbit IgG (S3731, 1:5000; Promega) or anti-mouse (S372B, 1:5000; Promega) antibodies.

### Detection of biological activity and quantification of CTB–EDIII fusion protein in transgenic plants

To detect the biological activity of CTB–EDIII produced in tomato plants, binding to monosialotetrahexosylganglioside (GM1) was detected using an enzyme-linked immunosorbent assay (ELISA). Nunc Maxisorp 96-well ELISA plates were coated with 3 µg/mL of GM1 (Sigma-

Aldrich, St. Louis, MO, USA) in bicarbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 25 mM NaHCO<sub>3</sub>, pH 9.6) and incubated at 4 °C overnight. After blocking with 1 % BSA in PBS, triplicates samples including recombinant CTB as standard protein were added to the wells in twofold serial dilutions and incubated for 2 h at 37 °C. The plates were then washed and probed with the same antibodies used for immunoblot analysis. The ELISA plates were developed by adding alkaline phosphatase buffer [10 % (v/v) diethanol amine, 0.1 % MgCl<sub>2</sub>, 0.02 % sodium azide, pH 9.8] with one tablet of phosphate substrate (S0942-100TAB, Sigma-Aldrich), and the absorbance was read at 405 nm. The concentration of assembled CTB–EDIII was extrapolated from the standard curve obtained with known amounts of CTB–antibody complex. The analysis of variance was performed using the Excel statistical analysis program (Microsoft, Redmond, WA, USA).

## Results

### Transformation and regeneration of transgenic plants

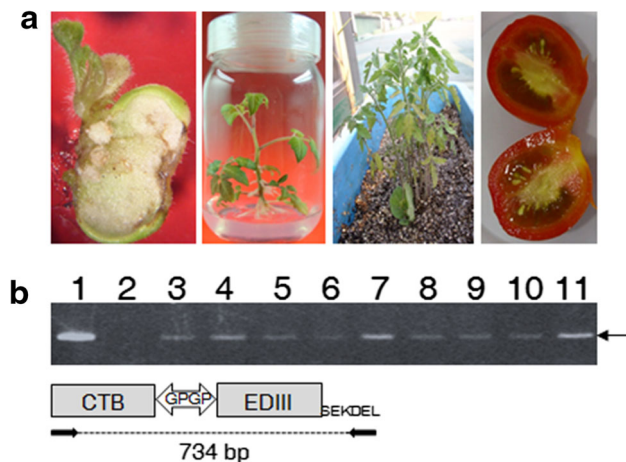
Tomato plants were transformed with *Agrobacterium* carrying the plant expression vector containing the CTB–EDIII gene construct. Kanamycin-resistant shoots formed at 4–5 weeks of growth on selection medium, and these shoots developed roots on MS hormone-free medium after 1 week. Figure 1a shows a typical transgenic shoot; root formation from a regenerated plant (in the bottle); maturation of plants transplanted to soil; and a transgenic tomato, respectively.

### Detection of CTB–EDIII fusion gene by genomic DNA PCR

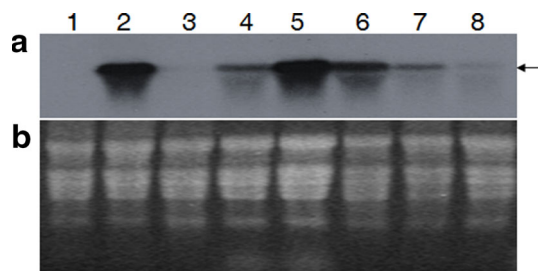
Integration of the fusion genes into the nucleus of tomato cells was verified by genomic DNA PCR amplification using gene-specific primers. The 734 nucleotide fragment corresponding to CTB–EDIII was detected in transgenic but not wild-type plants (Fig. 1b). These result indicated that the CTB–EDIII gene was successfully inserted into the DNA of tomatoes following *Agrobacterium*-mediated transformation.

### Detection of CTB–EDIII transcripts by northern blotting

The extracted RNA was analyzed for the presence of CTB–EDIII mRNA in transgenic plants. Positive signals for CTB–EDIII were detected in five of the seven plants analyzed, though there were variations in the intensity



**Fig. 1** Regeneration of transgenic tomatoes and detection of the chimeric gene: cholera toxin B subunit fused to dengue virus E glycoprotein domain III (CTB–EDIII). **a** Regeneration of transgenic plants. The results show shoot induction on selection medium containing kanamycin; root formation from a regenerated plant (*in the bottle*); maturation of plants transplanted to soil; and growth of transgenic tomato. **b** Detection of CTB–EDIII fusion gene by genomic DNA polymerase chain reaction (PCR) in transformed plants. CTB–EDIII fused with the endoplasmic reticulum (ER) retention peptide (SEKDEL) was detectable at the expected size (734 bp) (indicated by an *arrow* beside the figure). *Lane 1* plasmid DNA containing CTB–EDIII gene; *lane 2* genomic DNA from wild-type plant; *lanes 3–11* genomic DNA from transformed plants

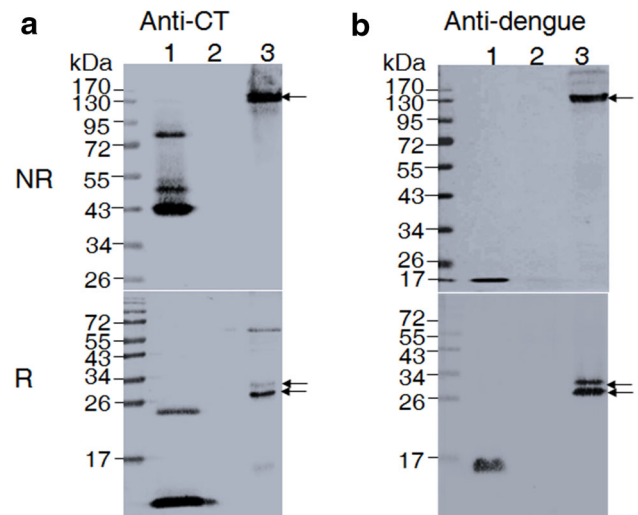


**Fig. 2** Detection of CTB–EDIII transcripts in transgenic plants. Northern blot analysis to detect CTB–EDIII transcripts. The expression of mRNA was confirmed at the expected size, indicated by an *arrow* (a). Total RNA separated on an agarose gel was stained by ethidium bromide (b). *Lane 1* total RNA extract from wild-type plant; *lanes 2–8* total RNA extracts from transgenic plants

(Fig. 2a). The highest-expressing transgenic plant was selected for further protein analysis. Non-transformed wild-type plants showed no mRNA expression.

#### Detection of CTB–EDIII fusion protein by immunoblotting

The synthesized and assembled fusion proteins were analyzed by Western blotting. The protein band at 130 kDa corresponding to the pentameric form was detected by both

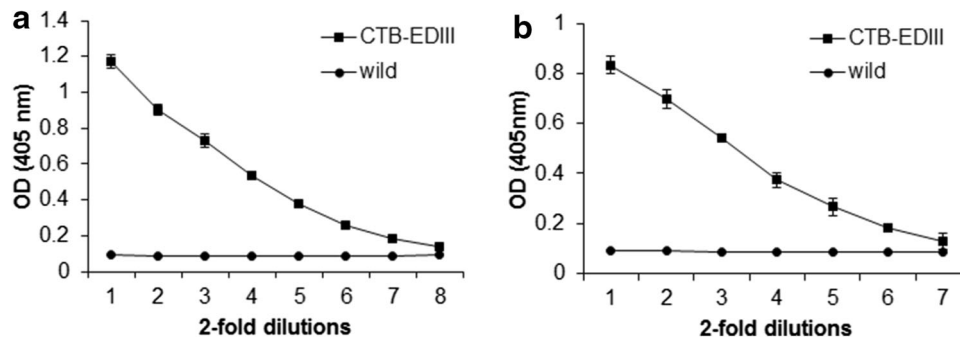


**Fig. 3** Detection of CTB–EDIII fusion protein in transgenic plants. Immunoblot analysis to detect the expression of CTB–EDIII under non-reducing (NR) or reducing (R) conditions. The results show a major protein band detected at 130 kDa under NR conditions and two monomeric forms at approximately 27 and 30 kDa under R conditions. Recombinant CTB protein (a) or dengue EDIII protein (b) purified from *E. coli* was used as positive control and wild-type plants as the negative control. The proteins were detected by anti-CT antiserum (a) and anti-dengue monoclonal antibodies (b). The expected band sizes are indicated by *arrows*. Molecular weight markers (in kDa) are shown on the *left*

anti-CT and anti-dengue antibodies under NR conditions (Fig. 3, upper panels). These molecules were disassembled under R conditions giving rise to two protein bands at 26 and 30 kDa (Fig. 3, lower panels). An additional protein band at 60 kDa was detected by anti-CT antiserum under R conditions, likely corresponding to dimeric fusion protein. These results showed that CTB–EDIII protein introduced into tomato plants was successfully synthesized and formed molecular pentamers.

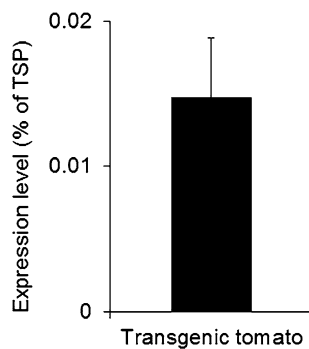
#### Detection of biological activity and quantification of CTB–EDIII

The concentration and biological activity of the fusion protein was assessed in a GM1-binding ELISA assay. The binding to GM1 ganglioside was detected by both anti-CT and anti-dengue antibodies, though stronger signals were detected with anti-CT than anti-dengue antibodies (Fig. 4). In contrast, wild-type plants showed no binding. These results indicated that the CTB–EDIII fusion protein was properly assembled in transgenic tomato plants and was biological active, as demonstrated by binding to GM1 ganglioside, which is present on epithelial cells of the intestine. The expression level of the fusion proteins was found to be 0.015 % of the TSP (Fig. 5).



**Fig. 4** Determination of monosialotetrahexosylganglioside (GM1)-binding ability of CTB–EDIII produced in transgenic plants. GM1 enzyme-linked immunosorbent assay (ELISA) to detect a functional CTB–EDIII protein. The results show the CTB–EDIII bound to GM1-

ganglioside. Protein extracts were diluted from 0.4 mg/mL of total soluble protein (TSP) for anti-CT antibody detection (a) or 0.8 mg/mL of TSP for anti-dengue antibody detection (b). Results are shown as the means of triplicate measurements  $\pm$ SD



**Fig. 5** Quantification of CTB–EDIII fusion protein in transgenic plants. The level of biologically active CTB fusion proteins showing binding affinity to GM1. The amount of protein, calculated by comparing with known amounts of bacterial CTB–antibody complex, was found to be  $\sim$ 0.015 % of TSP in plants. Results are shown as the means of three measurements  $\pm$ SD

## Discussion

Dengue is an important viral infection producing a wide spectrum of illnesses ranging from mild dengue fever (DF) to potentially fatal symptoms such as DHF or DSS. Dengue is rapidly spreading worldwide from tropical and subtropical endemic regions owing to globalization, urbanization and climate change. This situation can be mitigated by proper treatment and a universal vaccine (Bennett et al. 2010; Messina et al. 2014). According to the World Health Organization (WHO) report in 2011, 100 million cases of DF and half a million cases of DHF occur annually, with an average case fatality rate of  $\sim$ 5 % (Gurugama et al. 2010). Dengvaxia<sup>®</sup>—a live, attenuated, tetravalent recombinant yellow fever-17D—dengue virus vaccine—has been licensed recently in Brazil, Mexico and the Philippines for use in individuals 9–45 years of age. The results of the vaccine efficacy trials performed in Asia and Latin America indicated 60.8 % overall efficacy against

symptomatic dengue disease (Initiative 2015). A follow-up study indicated that there was an increased risk of hospitalization in individuals belonging to the under 9-year-old group (Initiative 2015). This increased risk is problematic because children are the most important age-group in terms of reducing the infection, as up to 90 % of patients with DHF are children less than 15 years of age (Gurugama et al. 2010). This outcome is most likely due to ADE as the vaccine formulation comprised four attenuated chimeric viruses from each serotype of dengue virus. Therefore, a global dengue vaccine for use in all age-groups is necessary for reducing the dengue mortality by 50 % and morbidity by 25 % by year 2020, as set out by the WHO (2009). Ideally, the vaccine should be efficient, inexpensive and safe, and should be easily applicable to young children. A plant-based mucosal (oral) vaccine could potentially meet these criteria.

Plants have often been used as delivery vehicles for testing mucosal vaccine candidates against human infections such as HBV, HIV, HPV, influenza virus, *Mycobacterium tuberculosis* and *Plasmodium falciparum*. In particular, edible plant vaccines delivered in the form of potato, lettuce and carrot have been explored for oral delivery by several research groups (Pniewski et al. 2011; Uvarova et al. 2013; Youm et al. 2010). Such vaccines were shown to be immunogenic in mice, and the antigens often formed particulate complexes such as virus-like particles (VLPs), which mimic authentic virions (Huang et al. 2005; Mason et al. 1996; Tacket et al. 2000). HPV antigen (HPVL1-E6/E7) expressed in tomatoes showed immunogenicity by inducing neutralizing antibodies and inhibiting tumor growth in mice (Monroy-Garcia et al. 2014). In this study, tomatoes were used not only to express dengue antigen fusion protein, but also to develop an oral vaccine against dengue infection.

Tomato is one of the most popular and valuable agricultural products in dengue-endemic areas, such as Africa

and Asia. Tomato is particularly amenable for production of vaccines because it contains two important substances: alpha tomatine (an endogenous plant adjuvant) and tomato lectin (an immunogen by forming tomato lectin/microbial antigen complex) (Carreno-Gomez et al. 1999; Morrow et al. 2004). Alpha tomatine, a saponin contained in green tomatoes at high concentrations, can induce a strong immune response at low doses (Oda et al. 2000; Sun et al. 2007). Hwang et al. reported successful oral vaccination with hemagglutinin H5 antigen protein purified from transgenic plants plus 10 µg of adjuvant saponin. The vaccine induced protection against a highly pathogenic avian influenza virus in immunized mice (Lee et al. 2015). Thus, we hypothesize that transgenic tomato plants could similarly induce a strong immune response and protect against dengue infection by oral administration of green tomatoes containing CTB–EDIII fusion protein antigen.

Although oral immunization normally induces immunogenic tolerance, this can be overcome by employing various strategies including the use of exogenous adjuvants such as AB5 toxins and saponins, the application of genetic-encoded particles such as VLPs, or the use of receptor-targeting ligands (Kim et al. 2010a; Monroy-Garcia et al. 2014; Tochikubo et al. 1998). CTB, a proven adjuvant (Boyhan and Daniell 2011; Harokopakis et al. 1998; Mikschofsky et al. 2009), is also used as a ‘breaker’ of immunity in autoimmune diseases (Stratmann 2015). In a previous study, we demonstrated oral immunogenicity against a fusion protein comprised of CTB and porphyromonas gingivalis fimbrial protein antigen (expressed in *E. coli*) (Kim et al. 2009), and evaluated its oligomerization and biological activity when expressed in plants (Kim et al. 2010b, 2013). We found that the orally administrated CTB fusion proteins could be internalized by M cells and enterocytes in Peyer’s patches (Kim et al. 2013).

In the present study, CTB was genetically fused to dengue antigen and directed to the ER for expression. The fusion protein was expressed in tomato plants, and we hypothesized that consuming the tomato would enhance the oral immune response by increasing the chance of interaction with GM1 ganglioside on epithelial cells of the gut. The assembled CTB–EDIII fusion protein was verified by immunoblotting and GM1 ELISA assays. The 130-kDa expression product corresponding to the pentameric form was detected under NR conditions. Two major protein bands were observed under R conditions (Fig. 3), likely corresponding to non-glycosylated and glycosylated monomers (as there are two *N*-glycosylation sites in the CTB sequence). The CTB–EDIII protein in the ER was largely intact, and evidence of slight degradation was observed in reduced samples by anti-CT antiserum (Fig. 3a) but not by anti-dengue antibodies (Fig. 3b). These

results suggested that the CTB–EDIII fusion protein could be stably expressed and assembled efficiently into pentamers in the ER. The CTB–EDIII protein showed a strong affinity for GM1-ganglioside, which is an important characteristic for a potential oral immunogen. Although the expression level of biologically active CTB–EDIII was relatively low, at 0.015 % of TSP in fresh plants, we anticipate that much higher yields could be achieved in freeze-dried green tomato fruits. Therefore, we expect that this transgenic tomato-derived CTB–EDIII could induce a strong immune response against dengue antigen by oral vaccination.

This study showed that CTB–EDIII could be expressed and assembled into a biologically active form in transgenic tomato plants. These transgenic tomatoes will be evaluated for their immunogenic potential in mice as an oral vaccine candidate against dengue infection.

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