

# M Cell-Targeting Ligand and Consensus Dengue Virus Envelope Protein Domain III Fusion Protein Production in Transgenic Rice Calli

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**Abstract** The spread of dengue (DEN) virus is becoming a major concern due to the possibility of primary infection with one of the four dengue serotypes (DEN 1–4) and secondary infection with other heterotypes, which can further aggravate clinical manifestations. A gene encoding consensus envelope protein domain III (cEDIII) of dengue virus with neutralizing activity against four dengue virus serotypes was fused to M cell-targeting peptide ligand (Co1) to increase its mucosal immunogenicity and was introduced into rice calli under the control of the inducible rice amylase 3D promoter expression system. The integration and expression of scEDIII–Co1 fusion gene in transgenic rice calli were confirmed by genomic DNA PCR amplification, Northern and Western blot analyses, respectively. The deliveries of cEDIII–Co1 fusion proteins into mucosal immune inductive site (including M cells) were confirmed by in vitro and in vivo antigen uptake assays. These results showed that plant-produced M cell-targeting peptide ligand, Co1, fusion antigen proteins have

the potential to be targeted to the mucosal immune system for improvement of immune responses.

**Keywords** Dengue virus · Tetravalent · M cell-target peptide ligand · Plant-based edible vaccine

## Introduction

Dengue is a flaviviral disease transmitted to humans via infected *Aedes* mosquitoes in tropical and subtropical areas. Recently, it has become a significant public health problem, transcending geographic boundaries and placing nearly 50 % of the global population at risk [1]. Because of the potential and considerable economical burden, advanced studies are needed to produce effective vaccines against dengue.

The properties of the dengue virus E protein from a vaccine point of view are associated with the host cell receptor-binding motif of domain III (EDIII). Four antigenically different strains make it difficult to develop an effective dengue vaccine because infection with just one of the dengue virus serotypes can lead to the full spectrum of dengue symptoms: fever, potentially life-threatening dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) after heterologous consecutive infections. To overcome this hurdle, the tetravalent types of dengue vaccine are mainly considered to provide protection against all serotypes without the antibody-dependent enhancement (ADE) of dengue virus infection [2, 3]. The consensus sequence was deduced by alignment of amino acid sequences of EDIII from different isolates of the four dengue viral serotypes. Mice immunized with the recombinant consensus envelope domain III (cEDIII) developed neutralizing antibodies against all four serotypes of dengue virus [4, 5].

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Since the first demonstration of the ability to express a vaccine antigen in 1990 [6], plant-based production systems offer safe and inexpensive vaccines with the capacity to deliver antigens to mucosal immune targets by oral vaccination [7–9]. However, a bottleneck against expanded vaccine production remains due to the low antigen yields related to oral tolerance and low immune responses and should ideally be resolved before being used as an edible vaccine. One strategy to increase immune responses in mucosal immune systems is the fusion of antigen proteins with ligands, which have an ability to deliver the fused antigen protein into mucosal immune systems for enhancing antigen uptake into mucosal immune cells. The representative ligands in transgenic plants were cholera toxin B subunit (CTB) and enterotoxigenic *E. coli* enterotoxin B subunit (LTB). Heat-labile toxin (LT), produced by *E. coli*, and cholera toxin (CT), produced by *Vibrio cholera*, are members of the AB<sub>5</sub> toxin family. CT is composed of a toxigenic A subunit and a non-toxic B subunit. The non-toxic B subunit possesses a pentameric conformation with a donut shape and binds to the G<sub>M1</sub>-ganglioside present on all nucleated mammalian cells [10]. CTB is one of the major candidates as an oral delivery carrier system for chemically or genetically coupled various vaccine-relevant antigens [11, 12]. Recent research efforts have shown functional activity of CTB or LTB fusion proteins [13–15]. Recently, Kim et al. [16] have shown that the M cell-targeting peptide ligand, Co1, in orally treated mice enhanced the uptake of fused antigen into the effective sites of mucosal immune systems and immune responses against fused antigen compared to antigens only.

In this study, the rice codon-optimized, synthetic cEDIII gene that allows protection against all four serotypes as a tetravalent type of dengue virus was fused to the M cell-binding peptide, Co1, to develop a plant-based edible vaccine with the potential for increased mucosal immune responses. The cEDIII–Co1 fusion proteins were produced under the control of the rice amylase 3D (RAmy3D) promoter, a strong inducible promoter, under a sugar starvation condition within a rice cellular expression system. The biologic functionality of cEDIII–Co1 fusion proteins for binding on mucosal immune cells was investigated in a mouse model.

## Materials and Methods

### Construction of Plant Expression Vectors

A gene encoding consensus dengue virus envelope protein domain III (103 aa, scEDIII), which was modified based on plant optimized codon usage [17] and with cross-neutralizing activity against four dengue virus serotypes [4], was

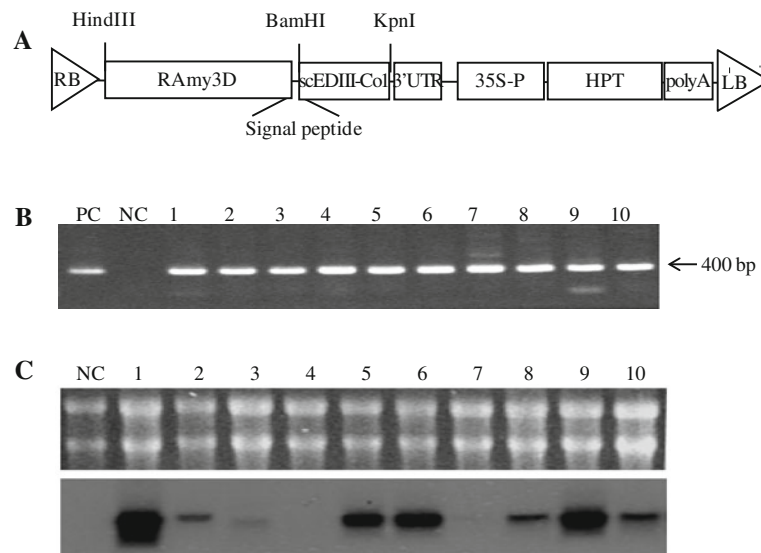
genetically fused to the M cell-binding peptide, Co1. The scEDIII gene fused with the M cell-binding peptide, Co1, was amplified from pMY657 [17] with gene-specific primers (forward primer scEDIII-F: 5'-GGA TCC AAG GGC ATG TCC TAC-3' and reverse primer scEDIII–Co1-R: 5'-GGT ACC TGG AAG TGG AGA TCT AGC TGG AAG CTG ATG AAA AGA GGA GGA GCC CTT CTT GAA-3'). The primers contained BamHI and KpnI sites (underlined) for easy subcloning, and the reverse primer contains the Co1 sequence (bold). PCR reaction contained 10 ng pMY657, 10 pmol primers, 200 μM dNTPs, 2 μL 10× Taq polymerase buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, and 0.1 % Triton X-100), 1.5 mM MgCl<sub>2</sub>, and 2U i-Taq polymerase (iNtRON Biotechnology, Seoul, Korea) in a total volume of 20 μL. The PCR reaction was conducted with the following PCR condition: 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and 1 cycle at 72 °C for 5 min. The PCR product was cloned into a pGEM-T Easy vector (Promega, Madison, WI) and its sequence was confirmed by DNA sequence analysis. The BamHI and KpnI-digested DNA fragment was subcloned into the same sites of pMYV657, which contains the promoter with signal peptide and the 3' untranslated region (3'UTR) of rice amylase 3D gene, resulting in pMYV685 (Fig. 1).

### Rice Callus Transformation

Rice calli (*Oryza sativa* L. cv. Dongin) were prepared and transformed via particle bombardment-mediated transformation [18] with pMYV685. After 3–5 days, the rice calli were transferred to N6 selection media supplemented with 2,4 dichlorophenoxyacetic acid (2 mg/L), sucrose (30 g/L), proline (0.5 g/L), glutamine (0.5 g/L), casein enzymatic hydrolysate (0.3 g/L), gelite (2 g/L), and hygromycin B (50 mg/L) as the antibiotic for selection at 2–3 weeks.

### Genomic DNA PCR Amplification

Genomic DNA in putative transgenic and non-transgenic rice calli was purified using the ZymoBead<sup>TM</sup> Genomic DNA Kit (Zymo Research, Orange, CA) and used for genomic DNA PCR analysis. The scEDIII–Co1 fusion gene was identified using scEDIII-F and scEDIII–Co1-R primers. The PCR mixture contained 100 ng of genomic DNA, 10 pmol primers, 200 μM dNTPs, 1× Taq polymerase buffer (1 mM Tris–Cl, pH 8.8, 5 mM KCl, and 0.01 % Triton X-100), 1.5 mM MgCl<sub>2</sub>, and 2 U i-Taq polymerase (iNtRON Biotechnology, Seoul, Korea) in a total reaction volume of 20 μL. Plant expression vector pMYV685 plasmid DNA (50 ng) were used as positive controls. The amplified DNAs were analyzed by 1.0 % agarose gel electrophoresis.



**Fig. 1** Plant transformation. The consensus domain III (scEDIII) of dengue virus glycoprotein fused with the M cell-targeting peptide, Co1 (pMYV685), was constructed into a plant expression vector under the control of a promoter (RAmy3D) with signal peptide and the 3' untranslated region (3'UTR) of rice amylose 3D gene (a). The scEDIII–Co1 fusion genes were detected by genomic DNA PCR amplification with target gene-specific primers in putative transgenic rice calli. Lane PC is plant expression vector containing cEDIII–Co1

fusion genes as positive control. Lane NC is non-transgenic rice callus as negative control. Lanes 1–10 are transgenic rice calli (b). The total RNA extracts from transgenic rice calli after induction via sugar starvation were subjected to Northern blot analysis to detect the transcripts of scEDIII–Co1 fusion gene. Lane NC is the total RNA extract of non-transgenic rice calli used as a negative control. Lanes 1–10 are the total RNA extracts of transgenic rice calli (c)

### Northern Blot Analysis

Total RNA was extracted from non-transgenic and transgenic rice calli tissues (500 mg) 5 days after induction under the condition of sugar starvation using Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the supplier's instructions. Thirty micrograms of total RNA were fractionated on 1.2 % formaldehyde-containing agarose gels and then transferred to a Hybond-N<sup>+</sup> membrane (Amersham–Pharmacia Biotech, Piscataway, NJ). The blot was hybridized overnight with a <sup>32</sup>P-labeled random-primed (Promega) scEDIII–Co1 probe at 65 °C in a modified church buffer (pH 7.4) that contained 1 mM EDTA, 250 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1 % hydrolyzed casein, and 7 % SDS in a Hybridization Incubator (Finemould Precision Ind., Seoul, Korea). The blot was washed twice with 2 × SSC plus 0.1 % SDS and twice with 2 × SSC plus 1 % SDS for 15 min each at 65 °C. Hybridized bands were detected by autoradiography using X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

### Western Blot Analysis and Quantification of Ligand Fusion Protein

Non-transgenic and transgenic rice calli were analyzed to detect cEDIII–Co1 fusion proteins by Western blot analysis. Transgenic rice calli (200 mg) were extracted 7 days after

induction under the condition of sugar starvation with extraction buffer (200 mM Tris–Cl, 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). Thirty micrograms of total soluble proteins (TSP) as determined by the Bradford protein assay (Bio-Rad, Inc., Hercules, USA) were separated by 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 2–2.5 h after boiling for 5 min in Tris–glycine buffer (25 mM Tris–Cl, 250 mM glycine, pH 8.3, and 0.1 % SDS). The separated protein bands were transferred from the gel to a Hybond C membrane (Amersham Pharmacia Biotech RPN303C) using a Mini Trans-Blot apparatus (Bio-Rad) at 150 mA for 2 h. Non-specific antibody binding was blocked by 5 % non-fat dry milk in a TBS buffer (20 mM Tris–Cl, pH 7.5, and 500 mM NaCl), followed by washing in a TBS buffer for 5 min. The membrane was incubated for 2 h in a 1:2,500 dilution of mouse anti-dengue virus antibody (Serotech, Oxford, UK) in a TBST antibody dilution buffer (TBS with 0.05 % Tween-20 and 2 % non-fat dry milk), followed by three washes in a TBST buffer (TBS with 0.05 % Tween-20). The membrane was incubated for 2 h in a 1:5,000 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma). The membrane was washed twice in the TBST buffer and once in a TMN buffer (100 mM Tris–Cl, pH 9.5, 5 mM MgCl<sub>2</sub> and 100 mM NaCl). After washing, color was allowed to develop using premixed BCIP/NBT solution (Sigma).

The transgenic rice calli lines showing high expression of cEDIII–Co1 fusion proteins in Western blot analysis were applied to suspension culture. The transgenic rice calli were harvested 7 days after induction under the condition of sugar starvation. The expression level of cEDIII–Co1 fusion protein in transgenic rice calli was measured by quantitative enzyme linked immunosorbent assay (ELISA). The concentration of protein extracts from transgenic calli using bicarbonate buffer, pH 9.6 (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35 mM NaHCO<sub>3</sub>), was determined by Bradford protein assay. The quantitative ELISA was conducted as previously described [19]. Briefly, the serial dilutions of plant protein extracts were coated at 100 µL/well into a 96-well microtitre plate (Thermo Fisher Scientific, Denmark) along with purified bacterial cEDIII. The coated plate was covered with Saran wrap and incubated overnight at 4 °C. The wells were blocked by adding 300 µL/well of 1 % bovine serum albumin (BSA) in PBS and incubated at 37 °C for 2 h, followed by three washes with PBST buffer (PBS containing 0.05 % Tween-20). The wells were loaded with 100 µL/well of a 1:4,000 dilution of mouse anti-dengue virus antibody and incubated for 2 h at 37 °C, followed by washing the wells three times with PBST buffer. The plate was then incubated with 100 µL/well of secondary antibody, a 1:7,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), for 2 h at 37 °C and washed three times with PBST buffer. The plate was incubated for 15 min at room temperature with 100 µL/well of phosphatase substrate (Sigma S0942). The plate was read at 405-nm wavelength in an ELISA reader (Packard Instrument MRA-006, Meriden, CT). The expression levels were estimated based on the known amounts of purified bacterial cEDIII using Excel 2007 program (Microsoft) and expressed as mean ± standard deviation of three independent experiments. To prepare bacterial cEDIII protein, scEDIII gene was constructed into pCold II vector [20] and expressed in *E. coli* strain BL21-RPIL. The recombinant cEDIII proteins was purified using an nickel column (Ni–NTA, Invitrogen, Carlsbad, CA) under denaturation conditions in 8 M urea. The purified recombinant proteins were quantified by Bradford protein assay and used to make standard curve.

#### Propagation and Induction of Rice Cell Suspension Culture

Transgenic rice calli were propagated and cultured at 28 °C in the dark in a shaking incubator at a rotation speed of 110 rpm. The transgenic rice cell suspension cultures were maintained in 300-mL flasks using N6 medium containing 2 mg/L 2,4-D, 0.02 mg/L kinetin, and 3 % sucrose [21]. A 10 mL inoculum was transferred every 9 days for sub-culturing. In order to induce target gene expression under

the control of the RAmy3D promoter, the N6 medium was replaced by fresh N6 (-S) medium (without sucrose) at 10 % (weight of wet cells/volume of medium) density. The rice callus was collected after induction with sugar starvation by pouring through 2–3 layers of Miracloth (Calbiochem, La Jolla, CA).

#### In Vitro and in Vivo Antigen Uptake Assays

For in vitro ligand fusion antigen binding assay, a gut loop containing Peyer's Patches prepared from a male BALB/c mice (Charles River Technology, MA, through Orient Bio., Sungnam, South Korea) were washed in ice-cold PBS buffer, whole-mounted using 4 % paraformaldehyde and blocked with 2.5 % BSA, 0.1 % glycine, and anti-mouse CD16/32 antibody/PBS. The protein extracts from non-transgenic rice calli and transgenic rice calli expressing cEDIII [17] or cEDIII–Co1 fusion proteins (50 µg of cEDIII–Co1 fusion protein) were applied for 2 h for binding. The Peyer's patches were then stained with Alexa Flour 350<sup>®</sup> conjugated-WGA (Invitrogen, Carlsbad, CA), rhodamine-labeled UEA-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-dengue virus antibody followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (BD Bioscience, Franklin Lakes, NJ) and analyzed by confocal laser scanning microscopy (CLSM; LSM 510 META; Carl Zeiss, Thornwood, NY). To monitor in vivo antigen uptake, BALB/c mice that received protein extracts from non-transgenic rice calli or transgenic rice calli containing cEDIII–Co1 fusion protein were euthanized at 10 min after oral administration, and Peyer's Patches were excised from the small intestine. The Peyer's Patches were washed and whole-mounted using 4 % paraformaldehyde. After blocking with 2.5 % BSA, 0.1 % glycine and anti-mouse CD16/32 antibody/PBS, the specimens were stained with Alexa Flour 350<sup>®</sup> conjugated-WGA, rhodamine-conjugated UEA-1 and anti-dengue virus antibody followed by FITC-conjugated anti-mouse IgG and then analyzed by CLSM.

## Results

#### Construction of Plant Expression Vector and Genomic DNA PCR Analysis

The consensus EDIII (scEDIII) gene was deduced in accordance with the amino acid sequence containing cross-neutralizing activity against four dengue virus serotypes and synthesized based on plant optimized codon usage [17]. To overcome low immune responses and immune tolerance due to the low expression level of the target protein, the M cell-binding peptide ligand, Co1, was fused in the c-terminus of the scEDIII gene. The scEDIII–Co1 fusion genes were

constructed into the plant expression vector with the RAmy3D promoter expression system, which is the sucrose starvation-inducible promoter, pMYV685 (scEDIII–Co1) (Fig. 1a). The rice expression vectors were transformed into rice calli via particle bombardment-mediated transformation methods, and putative transgenic calli were selected under hygromycin pressure. Ten putative transgenic rice calli for the scEDIII–Co1 fusion gene appeared at 2–4 weeks after transformation. A DNA fragment corresponding in size to the scEDIII–Co1 fusion gene (400 bp) was amplified by genomic DNA PCR amplification with primer sets specific to the scEDIII–Co1 fusion genes in all transgenic rice calli. The bands for scEDIII–Co1 fusion gene were not detected in non-transgenic rice calli (Fig. 1b).

#### Expression of scEDIII–Co1 Fusion Gene in Transgenic Rice Calli

Transcription of the scEDIII–Co1 fusion gene was confirmed in transgenic rice calli using a [ $^{32}$ P]-labeled scEDIII–Co1 fusion gene probe by Northern blot analysis. The positive signals for scEDIII–Co1 transcripts were detected in 8 of 10 transgenic rice calli (Fig. 1c). Expression of the cEDIII–Co1 fusion protein was evaluated by Western blot analysis in 6 transgenic rice callus lines chosen because they showed strong signals in Northern blot analysis. The cEDIII–Co1 fusion protein was detected as 3 bands with slightly different sizes near 17 kDa using anti-dengue virus antibody (Fig. 2a). The size of the purified bacterial EDIII protein (*serotype 2*) expressed in the pRSET vector expression system was approximately 16 kDa. However, no band corresponding to the cEDIII–Co1 fusion protein was detected in transgenic callus line #2 or in non-transgenic rice callus protein extracts (Fig. 2a).

#### Quantification of Ligand Fusion Proteins

Transgenic rice callus lines #5, #6, and #9 showing high expression level of cEDIII–Co1 fusion protein according to Western blot analysis so they were selected for measurement of the expression level. The transgenic rice calli were harvested 7 days after induction under a sugar starvation condition. The expression level of the cEDIII–Co1 fusion protein expressed in transgenic rice calli was quantified by quantitative ELISA with known amounts of purified bacterial cEDIII protein as a standard. The expression levels of cEDIII–Co1 fusion protein were expressed as a percentage of total soluble protein (percentage of TSP) (Fig. 2b). The amount of rice-produced cEDIII–Co1 fusion protein was found to be between 0.27 and 0.73 % of TSP. The highest level of cEDIII–Co1 fusion protein expression was found in transgenic rice callus line #6 that was selected for in vitro and in vivo antigen uptake assays.

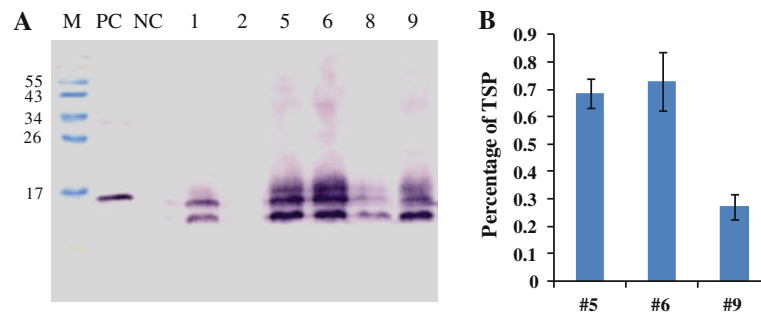
#### Binding of cEDIII–Co1 Fusion Proteins to M Cells in Peyer's Patches

M cell-specific targeting ability of the cEDIII–Co1 fusion proteins produced in transgenic rice calli was measured relative to the UEA-1<sup>+</sup>WGA<sup>−</sup> M cells by fluorescence microscopy. Transgenic rice calli protein extracts containing 50  $\mu$ g of cEDIII–Co1 fusion proteins were incubated into Peyer's Patches. Red color represents UEA-1<sup>+</sup>WGA<sup>−</sup> M cells bound only with rhodamine-labeled UAE-1 (red), purple color represents enterocytes bound with rhodamine-labeled UAE-1 (red) and Alexa Flour 350<sup>®</sup> conjugated-WGA (blue), yellow color represent cEDIII–Co1 fusion proteins (fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, green) bound to M cell (red). In both in vitro and in vivo assays, cEDIII–Co1 fusion proteins showed yellow and green colors together with yellow colors, indicating the binding of ligand fusion proteins to M cells on Peyer's Patches. There was no interaction of cEDIII alone or non-transgenic rice cell protein extracts with M cells or enterocytes in Peyer's Patches (Fig. 3).

#### Discussion

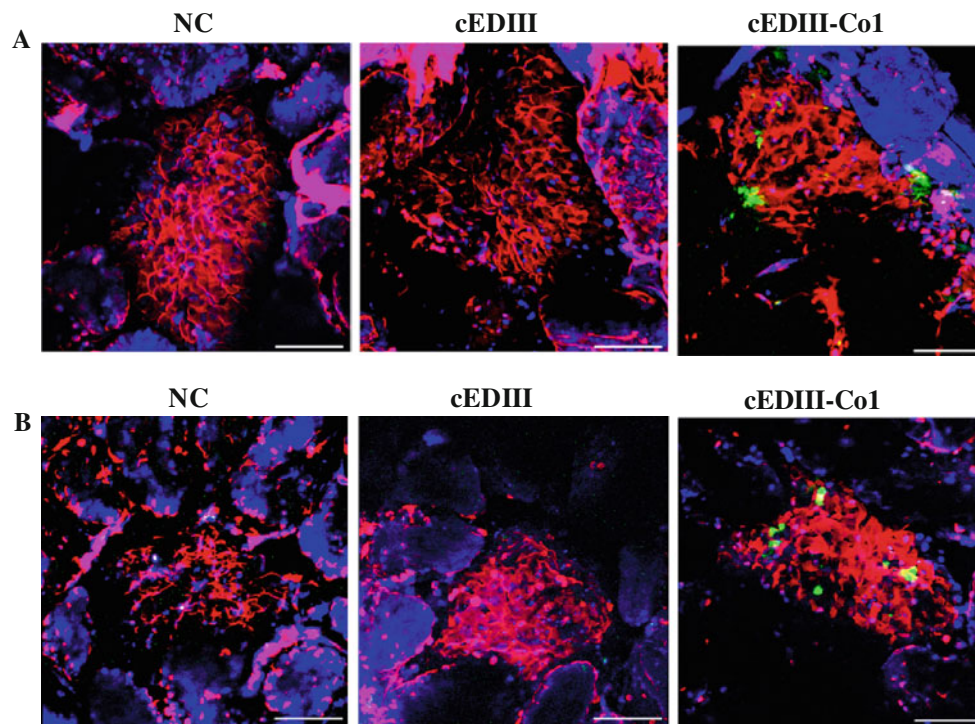
Development of plant-based vaccines has been facilitated because of the demand for safe and inexpensive vaccines. The success of plant-based oral vaccine development is responsible for the efficiency of sampling antigens and to present them to the mucosal immune system to induce high immune responses against target antigens. The small dose of antigens contained in transgenic plant materials, which is due to the low expression level of the antigen protein, provides a smaller opportunity for them to present their epitopes onto the antigen-presenting cells to efficiently induce immune responses and therefore results in low immune responses and oral tolerances [22, 23]. Many efforts to improve the expression level of target genes have been conducted using strong or tissue-specific promoters, codon optimization of target genes, transcriptional or translational factors with 5'UTR sequences, protein targeting to subcellular locations, and fusion to a stable protein like the B subunit of the *E. coli* heat-labile enterotoxin (LT) or cholera toxin (CT) [24].

An alternative strategy to overcome weak immune responses and immune tolerances is the use of ligands, which have the capacity to target the fused antigens into mucosal immune systems for improvement of antigen uptake and antigen presentation in antigen-presenting cells. For this purpose, the M cell-binding peptide, Co1, was fused to an antigen and introduced into rice calli. M cells in the mucosal immune system are specialized antigen sampling epithelial cells and are easily accessible to microorganisms



**Fig. 2** Western blot analysis and quantification of the cEDIII–Co1 fusion protein. The cEDIII–Co1 fusion proteins produced in transgenic rice calli after induction with sucrose starvation were separated via SDS-PAGE and subjected to Western blot analysis with anti-dengue virus antibody. Lane M is a prestained protein ladder (Fermentas, Glen Burnie, MD). Lane PC is purified EDIII (serotype 2) in *E. coli*. Lane NC contains non-transgenic rice callus protein

extracts used as a negative control. Lanes 1, 2, 5, 6, 8, and 9 are protein extracts in transgenic rice calli (a). The expression level of cEDIII–Co1 fusion protein in transgenic rice calli was measured 7 days after induction of sugar starvation condition by quantitative ELISA. Serial dilutions of bacterial cEDIII protein were used to create a standard curve to measure the expression level of cEDIII–Co1 fusion protein in transgenic rice calli (b)



**Fig. 3** Biologic activity of plant-produced cEDIII–Co1 fusion proteins in Peyer's Patches. The M cell-specific targeting abilities of the cEDIII–Co1 fusion proteins in transgenic rice calli were measured relative to UEA-1<sup>+</sup>WGA<sup>-</sup> M cells in vitro (a) and in vivo (b) assays by fluorescence microscopy. Rhodamine-labeled UEA-1 (red color)

bound to M cells, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG bound with ligand cEDIII fusion protein (green color). The combination (yellow color) of UEA-1 (red color) and cEDIII (green color) indicated the binding of ligand fusion proteins to M cells in the Peyer's Patches (Color figure online)

or migration molecules for tissue-specific consequences of lymphocyte priming in Peyer's Patches [10, 25]. Even though the mechanism of antigen uptake by M cells is not completely understood [26, 27], M cell-targeted antigen delivery approaches have been evaluated by several groups [10, 28], and surface markers for potential delivery of mucosal vaccines through the M cell have been identified

[29]. The binding ability of cEDIII–Co1 fusion proteins to Peyer's Patches was confirmed by M cell-binding assay in a mouse model. This result indicates that the cEDIII–Co1 fusion antigen protein can be delivered to the mucosal epithelium, including M cells, specialized epithelial cells responsible for antigen sampling, and successfully presented in antigen presenting cell of the mucosal immune system.

The cEDIII–Co1 fusion proteins in the Western blot analysis with anti-dengue virus antibody under the boiled condition were detected as three bands with slightly different and these results showed a similar band pattern as that observed in previous reports for the expression of foreign proteins in transgenic plants [30, 31]. Three bands shown in Western blot analysis may account for the presence of processed or unprocessed RAmy3D signal peptides because the secreted cEDIII–Co1 fusion protein into suspension culture medium representing processed RAmy3D signal peptide showed only one band (data not shown).

The expression levels of cEDIII–Co1 fusion proteins were measured in transgenic rice calli by quantitative ELISA. The expression level of cEDIII–Co1 fusion protein was approximately 0.73 % of the TSP in transgenic rice calli. The expression level showed considerably higher expression level compared to previous experiments; EDIII and CTB–EDIII protein produced in transgenic tobacco plants showed only 0.13–0.25 % of TSP based on Western blot analysis and 0.019 % of TSP based on the G<sub>M1</sub>-ELISA [14, 30]. The domain III protein of the dengue virus expressed in plants using a Tobacco mosaic virus (TMV) vector system was detected at 0.28 % TSP, and the purified domain III protein induced anti-dengue virus antibodies with neutralizing activity. Immunized mice with the plant-produced EDIII protein without an adjuvant did not elicit immune responses, but these responses were produced with an adjuvant [32]. It is expected that cEDIII–Co1 fusion proteins with high expression level and binding ability to the mucosal surface can be efficiently taken up into the mucosal immune system and improve immune responses against antigens to prevent infection with all dengue virus serotypes.

In this study, we wanted to develop a valuable plant-based oral vaccine against dengue virus infection. We constructed a plant expression vector using the plant codon-optimized consensus EDIII gene fused to the M cell-binding peptide, Co1, which was under the control of the RAmy3D promoter expression system and transformed in rice calli by the particle bombardment-mediated transformation method. The expression of cEDIII–Co1 fusion proteins was confirmed by Western blot analysis in transgenic rice calli. The plant-produced, cEDIII–Co1 fusion proteins showed binding activity to intestinal epithelial cells, including M cells of the gut-associated lymphoid tissues, by M cell-binding assays in a mouse model. These results suggest the feasibility of M cell-binding ligand, Co1 fusion protein use to improve immune responses due to antigen targeting of the mucosal immune system.

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## References

- Siddiqui, F. J., Haider, S. R., & Bhutta, Z. A. (2009). Endemic dengue fever: a seldom recognized hazard for Pakistani children. *Journal of Infection in Developing Countries*, 3, 306–312.
- Swaminathan, S., Batra, G., & Khanna, N. (2010). Dengue vaccines: state of the art. *Expert Opinion on Therapeutic Patents*, 20, 819–835.
- Miller, N. (2010). Recent progress in dengue vaccine research and development. *Current Opinion in Molecular Therapeutics*, 12, 31–38.
- Leng, C. H., Liu, S. J., Tsai, J. P., Li, Y. S., Chen, M. Y., Liu, H. H., et al. (2009). A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. *Microbes and Infection*, 11, 288–295.
- Chiang, C. Y., Liu, S. J., Tsai, J. P., Li, Y. S., Chen, M. Y., Liu, H. H., et al. (2011). A novel single-dose dengue subunit vaccine induces memory immune responses. *PLoS One*, 6, e23319.
- Curtiss, R. 3rd, & Cardineau, G.A. (1990) Oral immunization by transgenic plants. World patent application WO 90/02484. Washington university.
- Kim, T. G., Kim, B. G., Kim, M. Y., Choi, J. K., Jung, E. S., & Yang, M. S. (2010). Expression and immunogenicity of enterotoxigenic *Escherichia coli* heat-labile toxin B subunit in transgenic rice callus. *Molecular Biotechnology*, 44, 14–21.
- Mason, H. S., Ball, J. M., Shi, J. J., Jiang, X., Estes, M. K., & Arntzen, C. J. (1996). Expression of norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 5335–5340.
- Youm, J. W., Jeon, J. H., Kim, H., Min, S. R., Kim, M. S., Joung, H., et al. (2010). High-level expression of a human beta-site APP cleaving enzyme in transgenic tobacco chloroplasts and its immunogenicity in mice. *Transgenic Research*, 19, 1099–1108.
- Liljeqvist, S., Stahl, S., Andreoni, C., Binz, H., Uhlen, M., & Murby, M. (1997). Fusions to the cholera toxin B subunit: influence on pentamerization and GM1 binding. *Journal of Immunological Methods*, 210, 125–135.
- Kim, T. G., Huy, N. X., Kim, M. Y., Jeong, D. K., Jang, Y. S., Yang, M. S., et al. (2009). Immunogenicity of a cholera toxin B subunit *Porphyromonas gingivalis* fimbrial antigen fusion protein expressed in *E. coli*. *Molecular Biotechnology*, 41, 157–164.
- Boyhan, D., & Daniell, H. (2011). Low-cost production of pro-insulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant Biotechnology Journal*, 9, 585–598.
- Huy, N. X., Yang, M. S., & Kim, T. G. (2011). Expression of a cholera toxin B subunit-neutralizing epitope of the porcine epidemic diarrhea virus fusion gene in transgenic lettuce (*Lactuca sativa* L.). *Molecular Biotechnology*, 48, 201–209.
- Kim, T. G., Kim, M. Y., & Yang, M. S. (2010). Cholera toxin B subunit-domain III of dengue virus envelope glycoprotein E fusion protein production in transgenic plants. *Protein Expression and Purification*, 74, 236–241.
- Kim, M. Y., Kim, T. G., Yoo, H. S., & Yang, M. S. (2011). Expression and assembly of ApxIIA toxin of *Actinobacillus pleuropneumoniae* fused with the enterotoxigenic *E. coli* heat-labile toxin B subunit in transgenic tobacco. *Plant Cell Tissue and Organ Culture*, 105, 375–382.
- Kim, S. H., Seo, K. W., Kim, J., Lee, K. Y., & Jang, Y. S. (2010). The M cell-targeting ligand promotes antigen delivery and induces antigen-specific immune responses in mucosal vaccination. *Journal of Immunology*, 185, 5787–5793.
- Kim, M. Y., Yang, M. S., & Kim, T. G. (2012). Expression of a consensus dengue virus envelope protein domain III in transgenic callus of rice. *Plant Cell Tissue and Organ Culture*, 109, 509–515.

18. Chen, P. W., Lu, C. A., Yu, T. S., Tseng, T. H., Wang, C. S., & Yu, S. M. (2002). Rice alpha-amylase transcriptional enhancers direct multiple mode regulation of promoters in transgenic rice. *Journal of Biological Chemistry*, *277*, 13641–13649.
19. Kang, T. J., Kang, K. H., Kim, J. A., Kwon, T. H., Jang, Y. S., & Yang, M. S. (2004). High-level expression of the neutralizing epitope of porcine epidemic diarrhea virus by a tobacco mosaic virus-based vector. *Protein Expression and Purification*, *38*, 129–135.
20. Qing, G., Ma, L. C., Khorchid, A., Swapna, G. V., Mal, T. K., Takayama, M. M., et al. (2004). Cold-shock induced high-yield protein production in *Escherichia coli*. *Nature Biotechnology*, *22*, 877–882.
21. Thompson, J. A., Abdullah, R., & Cocking, E. C. (1986). Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Science*, *47*, 123–133.
22. Tremblay, R., Wang, D., Jevnikar, A. M., & Ma, S. (2010). Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. *Biotechnology Advances*, *28*, 214–221.
23. Neutra, M. R., & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature Reviews Immunology*, *6*, 148–158.
24. Lau, O. S., & Sun, S. S. (2009). Plant seeds as bioreactors for recombinant protein production. *Biotechnology Advances*, *27*, 1015–1022.
25. Takahashi, I., Nochi, T., Yuki, Y., & Kiyono, H. (2009). New horizon of mucosal immunity and vaccines. *Current Opinion in Immunology*, *21*, 352–358.
26. Cerutti, A., & Rescigno, M. (2008). The biology of intestinal immunoglobulin A responses. *Immunity*, *28*, 740–750.
27. Neutra, M. R., Mantis, N. J., & Kraehenbuhl, J. P. (2001). Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nature Immunology*, *2*, 1004–1009.
28. Tsuji, N. M., & Kosaka, A. (2008). Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends in Immunology*, *29*, 532–540.
29. Pielage, J. F., Cichon, C., Greune, L., Hirashima, M., Kucharzik, T., & Schmidt, M. A. (2007). Reversible differentiation of Caco-2 cells reveals galectin-9 as a surface marker molecule for human follicle-associated epithelia and M cell-like cells. *International Journal of Biochemistry and Cell Biology*, *39*, 1886–1901.
30. Kim, M. Y., Yang, M. S., & Kim, T. G. (2009). Expression of dengue virus E glycoprotein domain III in non-nicotine transgenic tobacco plants. *Biotechnology and Bioprocess Engineering*, *14*, 725–730.
31. Arakawa, T., Yu, J., Chong, D. K., Hough, J., Engen, P. C., & Langridge, W. H. (1998). A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nature Biotechnology*, *16*, 934–938.
32. Saejung, W., Fujiyama, K., Takasaki, T., Ito, M., Hori, K., Malasit, P., et al. (2007). Production of dengue 2 envelope domain III in plant using TMV-based vector system. *Vaccine*, *25*, 6646–6654.