Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–9, 2014

www.elsevier.com/locate/jbiosc

Expression and characterization of an M cell-specific ligand-fused dengue virus tetravalent epitope using *Saccharomyces cerevisiae*

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Received 27 February 2014; accepted 11 June 2014
Available online xxx

A fusion construct (Tet-EDIII-Co1) consisting of an M cell-specific peptide ligand (Co1) at the C-terminus of a recombinant tetravalent gene encoding the amino acid sequences of dengue envelope domain III (Tet-EDIII) from four serotypes was expressed and tested for binding activity to the mucosal immune inductive site M cells for the development of an oral vaccine. The yeast episomal expression vector, pYEGPD-TER, which was designed to direct gene expression using the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter, a functional signal peptide of the amylase 1A protein from rice, and the GAL7 terminator, was used to clone the Tet-EDIII-Co1 gene and resultant plasmids were then used to transform *Saccharomyces cerevisiae*. PCR and back-transformation into *Escherichia coli* confirmed the presence of the Tet-EDIII-Co1 gene-containing plasmid in transformants. Northern blot analysis of transformed *S. cerevisiae* identified the presence of the Tet-EDIII-Co1-specific transcript. Western blot analysis indicated that the produced Tet-EDIII-Co1 protein with the expected molecular weight was successfully secreted into the culture medium. Quantitative Western blot analysis and ELISA revealed that the recombinant Tet-EDIII-Co1 protein comprised approximately 0.1–0.2% of cell-free extracts (CFEs). In addition, 0.1–0.2 mg of Tet-EDIII-Co1 protein per liter of culture filtrate was detected on day 1, and this quantity peaked on day 3 after cultivation. *In vivo* binding assays showed that the Tet-EDIII-Co1 protein was delivered specifically to M cells in Peyer's patches (PPs) while the Tet-EDIII protein lacking the Co1 ligand did not, which demonstrated the efficient targeting of this antigenic protein through the mucosal-specific ligand.

[**Key words:** Oral feeding; M cell-specific peptide ligand; Dengue virus; Domain III of the dengue envelope protein; Baker's yeast]

Dengue viruses are enveloped, positive sense RNA viruses that are members of the Flaviviridae family. Dengue infection has been acknowledged as the most important arthropod-borne viral infection in humans. Dengue infections can reach up to 50–100 million cases worldwide annually with approximately 500,000 patients hospitalized for Dengue Hemorrhage Fever (DHF) and Dengue Shock Syndrome (DSS), with up to a 5% fatality rate in some areas (1,2). Recently, there has been a dramatic increase in the annual average number of DHF and dengue fever cases reported to the World Health Organization (WHO) (2). Dengue viruses exist as four distinct serotypes, which makes the development of an effective dengue vaccine a great challenge since the vaccine must be tetravalent (3). The requirement for a tetravalent vaccine is due to the likelihood that a secondary infection with a different viral serotype leads to the most severe forms of dengue virus infection such as DHF and DSS. This increase in disease severity is supposedly due to the presence of sub-neutralizing antibodies generated during the primary infection and their enhancement to viral infection (4,5). Although dengue infections have been considered a global public health priority, the development of a safe and effective dengue vaccine has been hampered by the strict requirements for tetravalency, the complex nature of the disease, and the lack of investment (2). As such, no proven vaccine has been developed to date.

The discovery of dengue envelope domain III (EDIII) as a neutralizing epitope (6–11) has opened up great opportunity for the development of a safe and effective recombinant dengue vaccine. Accordingly, several approaches have been attempted to overcome the tetravalency requirement. These included expression of a single tandem array of each EDIII serotype or a synthetic consensus protein based on the amino acid sequence of all four EDIII serotypes (12–16). Moreover, the immunogenic efficacy of recombinant tetravalent proteins has been confirmed (12,13), and their limitations, including the triggering of a balanced immune response, have been further improved through the expression of the protein in a eukaryotic host (17).

Progress in understanding the mucosal immune system has led to the development of various oral vaccines against infectious agents. Mucosal vaccines have several advantages over systemic vaccines: they require less strict regulation since the oral route is more tolerable to endotoxins as well as other impurities than the parenteral route; they do not require special storage (cold-chain free) or properly trained medical staff for delivery; they do not involve complications normally involved with parenteral vaccines that can lead to better compliance; and they provide mucosal as

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.06.005

well as systematic protection for recipients (18–20). Furthermore, mucosal vaccines have been shown to be advantageous for protection against not only mucosal transmitted infections but also non-mucosal transmitted infections such as malaria, hepatitis B, and Japanese encephalitis caused by a Flaviviridae virus (21).

A key consideration in the development of an oral vaccine is that the introduced antigen must pass through the gut wall where antigen sampling occurs by transcytosis in membranous or microfold cells (M cells). M cell-mediated transcytosis of an internalized antigen incites the initiation of both the antigen-specific mucosal immune response through secretory IgA production and the systemic immune response (22–24). Recent studies have shown effective mucosal immune induction through targeting antigens using M cell-specific antibodies, M cell-specific expression molecules, and M cell-specific ligands (22,25,26).

Baker's yeast, *Saccharomyces cerevisiae*, is an attractive heterologous vaccine expression system because it combines the advantages of simple prokaryotic systems, including high expression level, ease of scale-up, and genetic manipulation and culturing, with the inherent advantage of eukaryotic post-translational modifications and secretion. Moreover, *S. cerevisiae* is a generally recognized as safe (GRAS) organism and is known for its high-quality protein and vitamins levels, which allow for live and oral administration for pharmaceutical, livestock feed, and food applications. Due to the strong adjuvant properties of yeast derivatives, the use of yeast expression systems for recombinant vaccine formulation is desirable. Recently, a well-balanced immune response to dengue viruses was observed when a yeast expression system was used for the production of dengue virus epitope proteins (17).

In this study, *S. cerevisiae* was utilized to express an M cell-specific ligand fused-tetravalent tetrameric antigen created through a fusion of EDIIIs from all four Dengue serotypes in order to create an efficient and balanced mucosal immune response through oral administration. In addition, delivery of the fusion protein to mucosal immune inductive sites was assessed by *in vivo* antigen uptake assays.

MATERIALS AND METHODS

Strains and culture conditions Plasmids were maintained and propagated in *Escherichia coli* HB101 or DH5α according to standard procedures (27). *E. coli* strains were maintained in Luria–Bertani (LB) medium supplemented with appropriate antibiotics.

S. cerevisiae strain 2805 (*MATα pep4::HIS3 prb 1-δ Can1 GAL2 his3 ura3-52*) (28) was selected as the expression host for yeast recombinant proteins. *S. cerevisiae* strains were maintained on YEPD plates (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar). A uracil-deficient selective (ura$^-$) medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acids, 2% dextrose, and 1.5% agar) was employed for the screening of transformants at 30°C.

For expression cultures, a healthy transformant colony was inoculated into 5 ml ura$^-$ medium and incubated for 48 h at 30°C with continuous agitation (200 rpm). Subsequently, 250 μl of the primary inoculum was transferred into 5 ml of YEPD medium and cultured for 16 h at 30°C with continuous agitation (200 rpm). This 5-ml culture was then used as an inoculum for a 40-ml YEPD medium culture in a 300 ml Erlenmeyer flask. Expression cultures were grown at 30°C with continuous agitation (200 rpm), after which the cells and culture filtrates were harvested and examined for the expression of recombinant protein (17).

Construction of yeast expression plasmids and yeast transformation The tetravalent tetrameric EDIII (Tet-EDIII) protein was created by fusing the EDIIIs from all four serotypes in the order of serotype 1, 3, 4 and 2, which were joined using flexible pentaglycine peptide linkers, as described previously (Fig. 1A) (12). The genes encoding EDIII serotypes 1 and 3 were cloned from total RNAs extracted from Vero cells infected with dengue serotype 1 (GenBank no. JF967947) and 3 (GenBank no. JF968056), respectively. The EDIII gene of serotype 4 was obtained from serotype 4 isolate DENV-4/PH/BID-V3361/1956 (GenBank no. GQ868594). The EDIII gene from serotype 2 was obtained from the plasmid pMYV497 in the previous study (29). All EDIII genes were sequenced and subsequently used as templates for constructing the Tet-EDIII sequence by overlapping extension PCR. The resulting Tet-EDIII gene was sequenced and further fused with the M-cell specific peptide ligand (Co1) (22) through PCR using synthetic primers. The fused Tet-EDIII and Co1 gene (Tet-EDIII–Co1) was confirmed by DNA sequencing (Fig. 1B and C).

A rice amylase signal peptide sequence (RamylA) (30) was added at the 5′ termini of the encoding DNA sequences of Tet-EDIII and Tet-EDIII-Co1 by overlap extension PCR containing *Bam*HI and *Sal*I at their 5′ and 3′ termini, respectively, to direct the secretion of expressed protein. The resulting Ramy1A–Tet-EDIII and Ramy1A–Tet-EDIII-Co1 amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and confirmed by DNA sequencing, yielding the pTet and pTetCo1 plasmids, respectively. The pTet and pTetCo1 plasmids were digested with *Bam*HI and *Sal*I and the excised inserts were directionally cloned into the *Bam*HI/*Sal*I-digested yeast episomal shuttle vector pYEGPD-TER (30), which had the same restriction sites between the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and the galactose-1-P uridyl transferase (*GAL7*) terminator, yielding plasmids pYEGTet and pYEGTetCo1, respectively. The sequence information for each primer is provided in Table 1.

S. cerevisiae 2805 cells were transformed with the constructed recombinant plasmids using the LiAc method (31) and transformants were selected on ura$^-$ selective plates. The stability of the introduced plasmids in yeast was measured as described previously (27).

Analysis of expression Northern blot, Western blot, and ELISA analyses were used to detect the RNA expression and protein production of Tet-EDIII and Tet-EDIII-Co1. For Northern blots, total RNA extraction was carried out as described previously (32). RNA concentration was determined by UV spectrophotometry and 30 μg of total RNA was separated on 1.2% formaldehyde-agarose gels. The RNA was transferred to a nylon membrane (Hybond, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Hybridization was performed with modified Church buffer (250 mM Na_2HPO_4, 1 mM EDTA, 7% SDS, 0.17% H_3PO_4, and 1% hydrolyzed casein) with radioactive probes produced by the random primer method (Promega).

For Western blot analysis, cell-free extracts (CFEs) were prepared as described previously (33). In order to obtain concentrated culture filtrate, the culture media of recombinant *S. cerevisiae* were collected and then concentrated using 30 kDa cutoff Centricon column (Millipore, County cork, Ireland), dialyzed against PBS twice for 4 h at 4°C, and sterilized by passing through a 0.4-μm syringe filter (30). Protein concentration was determined by Bradford assay using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) (33). Sample aliquots of the CFE and culture filtrate were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto Hybond-C Extra nitrocellulose filter membranes

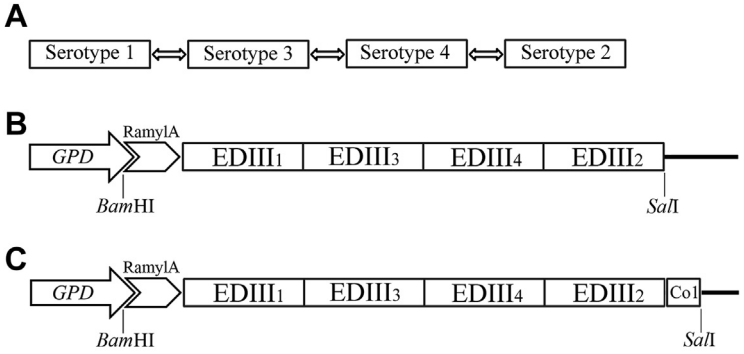

FIG. 1. Schematic diagram of Tet-EDIII protein structure (A) showing the order of each EDIII serotype with pentaglycine linkers (double headed arrows). The Tet-EDIII (B) and Tet-EDIII-Co1 (C) expression cassettes are also shown. The RamylA–Tet-EDIII and RamylA–Tet-EDIII-Co1 were constructed by overlap extension PCR and cloned into the episomal vector pYEGPD-TER using *Bam*HI and *Sal*I. Both constructs were expressed under control of the *GPD* promoter.

Please cite this article in press as: Nguyen, N.-L., et al., Expression and characterization of an M cell-specific ligand-fused dengue virus tetravalent epitope using *Saccharomyces cerevisiae*, J. Biosci. Bioeng., (2014), http://dx.doi.org/10.1016/j.jbiosc.2014.06.005

TABLE 1. List of primers used for construction of the Tet-EDIII and Tet-EDIII-Co1 expression cassette and envelop domain III antigen of four serotypes in *E. coli*. The underlined regions indicate the restriction enzyme sites.

Target gene[a]	Primer Orientation	Primer sequence	Reference
Ramy1A	Forward	AAGGATCCATGCAGGTGCTGAACACCA	17
	Reverse	CCCGGCTGTCAAGTTAGAGGA	17
	OL[b]-Reverse	AACTTGACAGCCGGGAAGGGGATGTCATAT	This study
Serotype 1	OL-Forward	ATATGACATCCCCTTCCCGGCTGTCAAGTT	This study
	Forward	AAGGGGATGTCATATGTGATG	17
	Reverse	GCTGCTTCCTTTCTTGAACCA	17
	OL-Reverse	ATAGCTCATCCCCTTTCCTCCTCCTCCTCCGCTGCTTCCTTTCTT	This study
Serotype 3	OL-Forward	AAGAAAGGAAGCAGCGGAGGAGGAGGAGGAAAGGGGATGAGCTAT	This study
	Forward	AAGGGGATGAGCTATGCAA	17
	Reverse	CGAGCTTCCCTTCTTGTAC	17
	OL-Reverse	AAGAAGGGAAGCTCGGGAGGAGGAGGAGGAAAGGGAATGTCATAC	This study
Serotype 4	OL-Forward	AAGAAGGGAAGCTCGGGAGGAGGAGGAGGAAAGGGAATGTCATAC	This study
	Forward	AAGGGAATGTCATACACGA	17
	Reverse	TCGTGTATGACATTCCCTT	17
	OL-Reverse	GTATGACATTCCTTTTCCTCCTCCTCCTCCGGAACTCCCTTTCCT	This study
Serotype 2	OL-Forward	AGGAAAGGGAGTTCCGGAGGAGGAGGAGGAAAAGGAATGTCATAC	This study
	Forward	AAAGGAATGTCATACTCTATG	29
	Reverse	AGAACTTCCTTTCTTGAACCA	29
Ramy1A—Tet-EDIII	OL-Reverse	CAAGTCGACTCAAGAACTTCCTTTCTTGAA	This study
Ramy1A—Tet-EDIII-Co1	OL-Reverse	CAAGTCGACTCATGGTAGTGGACTTCTAGCTGGTAGTTGATGGAAAGAAGAACTTCCTTTCTT	This study

[a] Genes are listed in order of an expression cassette. The underlined regions indicate the restriction enzyme sites.
[b] OL represents the primer used for the overlapping PCR.

(Hybond, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Primary antibodies (AbD Serotec, Raleigh, NC, USA) that recognize each serotype detected target proteins. After incubation with goat anti-mouse secondary antibody (Assay Designs, Ann Arbor, MI, USA), the membrane was washed three times with Tris buffered saline pH 7.5 containing 1% Tween 20 (TBST) and developed by BCIP/NBT in TMN buffer (100 mM Tris, pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl).

Quantitative ELISA determined the quantity of expressed recombinant proteins in CFEs and culture filtrates as described previously (30). In addition, quantitative Western blot analysis was conducted via side-by-side band intensity comparison using a densitometer.

Preparation of serotype antigens and antibodies Dengue EDIII antigens from each serotype, which were expressed in recommended *E. coli* hosts (BL21-CodonPlus-RIL for serotypes 2, 3, and 4 expression host: M15 for serotype 1 expression host), and the *E. coli*-expressed synthetic consensus EDIII (scEDIII) antigen were obtained from our previous study (17).

To prepare antibodies specific to serotypes 3 and 4, *E. coli*-expressed EDIIIs for serotype 3 and 4 were purified and injected subcutaneously into 8-week-old BALB/c mice (Charles River laboratory, Wilmington, MA, USA) using Freund's complete and incomplete adjuvant. Antisera were collected 4 days after the 3rd booster injection and checked for specificity against corresponding antigens as well as for cross-reactivity against other serotype antigens.

***In vivo* antigen uptake assay** An *in vivo* antigen uptake assay was performed as described previously (22). Briefly, the 3-day old recombinant strain culture filtrates were concentrated using 30-kDa cutoff Centricon Plus-70 columns. The final samples were concentrated by approximately 300 fold. BALB/c mice that were administered approximately 5 μg of either Tet-EDIII or Tet-EDIII-Co1 were euthanized 30 min after oral administration, and Peyer's patches (PPs) were excised from the small intestine. The PPs were then washed vigorously with ice-cold PBS containing 1% Tween and whole mounted using 4% paraformaldehyde at 4°C overnight. After blocking with 3% BSA, 0.1% glycine in PBS at room temperature for 2 h, the PP sections were incubated with anti-dengue (AbD Serotec, 1:100 dilution) and anti-M cell (NKM 16-2-4, 1:250 dilution) antibodies for 2 h at room temperature. After washing, anti-rat IgG-Alexa 680 (Invitrogen, Carlsbad, CA, USA, 1:500 dilution) was added and the samples were incubated at room temperature for 1 h. The samples were washed and stained with anti-mouse IgG-FITC (Sigma, St. Louis, MO, USA, 1:250 dilution), rhodamine-UEA-1, Alexa 350-WGA for 2 h at room temperature. Finally the samples were embedded in 30% glycerol and analyzed using a CLMS (25). A similar procedure was also applied to label the M cell complement receptor C5aR (22).

RESULTS

Construction of expression plasmids and transformation The structure of the recombinant Tet-EDIII protein is depicted in Fig. 1. The gene encoding Tet-EDIII protein was created by two rounds of overlap extension PCR. The resulting Tet-EDIII gene encoded the EDIIIs of all four serotypes in order of serotype 1, 3, 4, and 2 with corresponding sequences for pentaglycine linkers between each serotype. The Tet-EDIII gene was further manipulated by fusion of the M-cell specific ligand (Co1) at the C terminus and was cloned into a yeast expression vector. The resulting pYEGTetCo1 plasmid is a multi-copy episomal plasmid for the expression of the Tet-EDIII-Co1 gene, which harbors the secretion signal sequence of rice amylase 1A (*Ramy1A*) under the control of the *GPD* promoter. As a comparison, the pYEGTet plasmid was also constructed for the expression of the Tet-EDIII gene alone, lacking the M-cell specific ligand (Fig. 1).

Eleven candidate transformants for each construct were randomly selected from ura- selection medium and subjected to plasmid extraction followed by PCR amplification of the Tet-EDIII gene and back transformation into *E. coli* to confirm the presence of correct plasmids. For the Tet-EDIII-Co1 construct, all samples showed the correct 1427-bp PCR amplicon corresponding to the region from the *RamylA* sequence to the end of the Tet-EDIII-Co1 sequence. For the Tet-EDIII plasmid, an appropriate 1391-bp PCR amplicon was amplified from all transformants (data not shown). These results indicated that the expression plasmids existed in the transformants as an episomal plasmid. The stability of the transforming plasmid in the selected transformants revealed that more than 83% of the plated transformed cells maintained the plasmid up to 72 h after cultivation in non-selective liquid media. No abnormal transformant growth patterns due to the presence of the transforming plasmid were observed.

Expression of Tet-EDIII-Co1 and Tet-EDIII Northern blot analysis using RNA extracted from 3-day-old cultured yeast was used for analyzing the expression of Tet-EDIII-Co1 and Tet-EDIII. Northern blot analysis using the cloned Tet-EDIII gene as a probe revealed that all transformants expressed the corresponding Tet-EDIII-Co1 (Fig. 2A) and Tet-EDIII (Fig. 2B) transcripts. No significant variation in the level of transcription was observed among the Tet-EDIII transformants. However, the Tet-EDIII-Co1 transformants showed variation in the accumulation of Tet-EDIII-Co1 transcript, indicating that there is variation in copy number of the expression plasmid among the transformants. Interestingly, transcription of Tet-EDIII and Tet-EDIII-Co1 tended to correlate well with transcription of the glyceraldehyde 3-phosphate dehydrogenase (*GPD*) gene, which was used as an internal control in our Northern blot analysis.

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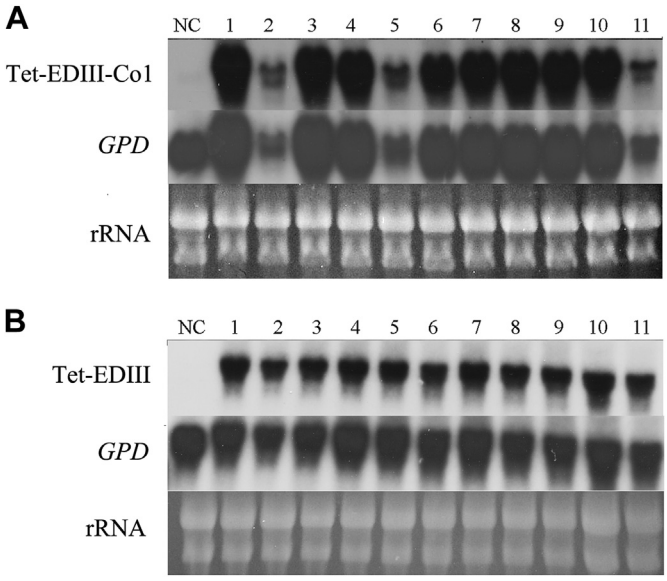

FIG. 2. Northern blot analysis of Tet-EDIII-Co1 (A) and Tet-EDIII (B) transformants. Total RNA of the mock transformant (vector only) was used as the negative control (NC). Lanes 1 to 11 contain total RNA preparations from 11 transformants. RNA samples were hybridized with radioactive Tet-EDIII gene as a probe. Glyceraldehyde-3-phosphate dehydrogenase (*GPD*) transcription is shown as internal control. Ribosomal (rRNA) is shown to confirm that an equal amount of RNA was loaded in each lane.

Transformants that showed a high Tet-EDIII-Co1 and Tet-EDIII expression level were selected for further experimentation and are referred to as TYEGTetCo1-1 and TYEGTet-4, respectively. The temporal expression pattern of the Tet-EDIII-Co1 and Tet-EDIII transcript was analyzed by Northern blot analysis using the cloned Tet-EDIII gene as a probe (Fig. 3). As shown in Fig. 3A, the accumulation of Tet-EDIII-Co1 transcript peaked at day 1 of cultivation and then decreased until only a small amount of transcript was observed after 5 days of cultivation. No difference in the expression pattern from that of the Tet-EDIII-Co1 transcript was observed for the Tet-EDIII transformant (Fig. 3B). In addition, correlation between the transcription level of the target gene and *GPD* gene was also observed, which could be ascribed to the expression characteristics directed by the same *GPD* promoter. This result agreed with our previous studies on expression in *S. cerevisiae* using a *GPD* promoter of an episomal pYEGPD-TER plasmid (30,34).

Immunoblot analysis of recombinant Tet-EDIII-Co1 and Tet-EDIII proteins Appropriate antigen presentation is an important step to induce immune response specially when using multivalent antigen. In order to determine the presence of EDIII from each serotype as one of four components in a tetravalent tetramer, it is desirable to have antibodies without significant cross-reactivity. Thus, we tested for the cross-reactivity of available antibodies against *E. coli*-expressed EDIII of each serotype (Fig. 4). Since the commercial monoclonal antibody MCA2277 (AbD Serotec) detected *E. coli*-expressed EDIIIs of serotypes 1 and 2 but not those of serotypes 3 and 4 under our experimental conditions (Fig. 4), mouse polyclonal antibodies against *E. coli*-expressed EDIIIs of serotypes 3 and 4 were obtained and tested for their cross-reactivity. As shown in Fig. 4, antibody against serotype 3 showed strong reactivity against serotype 3 (from which the antibody itself was made) and serotype 1, but cross-reactivity with serotypes 2 and 4 was not as distinctive as with serotypes 3 and 1. The strong cross-reactivity of serotype 3 antibody to serotype 1 antigen appears to be in good agreement in that serotype 3 shows the highest sequence identity (70.4%) to serotype 1 (see Supplementary Table S1). Antibody against serotype 4 showed strong reactivity to its own serotype 4 antigen, a strong but slightly weaker reactivity to serotypes 1 and 3, and a weak reactivity to serotype 2. However, no one serotype-specific antibody was obtained as the commercial monoclonal antibody MCA2277 reacted to two serotypes and antibodies against serotypes 3 and 4 reacted to all four serotypes, albeit with various intensity. Therefore, using currently available antibodies, it was hard to determine the presentation of serotype-specific EDIIIs in a tetravalent tetramer at this point. In addition, the *E. coli*-expressed synthetic consensus EDIII (scEDIII) protein from our previous study (17) showed considerably strong reactivity regardless of the antibody used. Therefore, we used the scEDIII protein as a control antigen for the quantification of target protein.

Western blot analysis using three different antibodies showed the presence of specific bands around 40 kDa in CFEs from Tet-EDIII-Co1 and Tet-EDIII transformants (Fig. 5B). More interestingly, side by side comparison of protein samples from Tet-EDIII-Co1 and Tet-EDIII transformants revealed that the size difference, due to the presence or absence of a 12 amino acid residue-long peptide ligand, was obvious enough to result in differential displaying on SDS-PAGE. The estimated molecular weight, antigen–antibody reaction, and the size difference due to the peptide ligand strongly suggested that the Co1 ligand-fused tetrameric tetravalent EDIII of all serotypes was successfully produced in *S. cerevisiae*. In addition, culture filtrates were also evaluated for the presence of recombinant Tet-

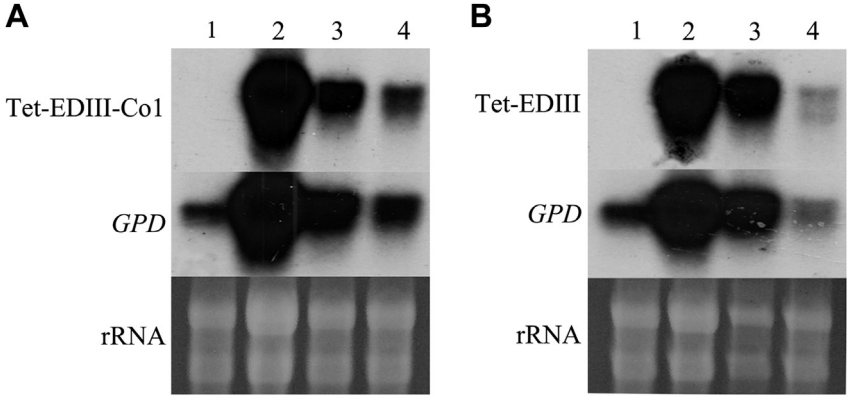

FIG. 3. Temporal expression analysis of Tet-EDIII-Co1 (A) and Tet-EDIII (B). Lane 1 contains total RNA of 3-day-old culture of the mock transformant. Lanes 2 to 4 contain total RNA of 1-, 3-, and 5-day-old cultures of the Tet-EDIII-Co1 transformants (A) and Tet-EDIII transformants (B). RNA samples were hybridized with radioactive Tet-EDIII gene as a probe. *GPD* transcription is shown as internal control. rRNA is shown to confirm that an equal amount of RNA was loaded in each lane.

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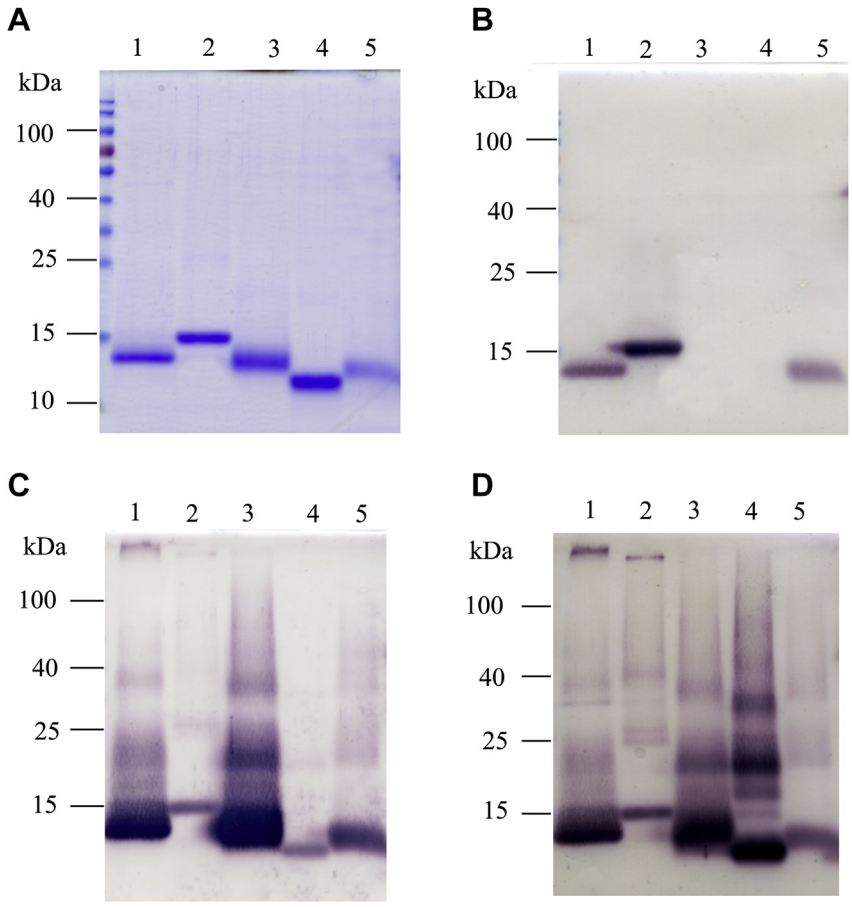

FIG. 4. Western blot analysis of *E. coli*-expressed EDIII of each serotype using three different antibodies. A Coomassie blue-stained SDS-PAGE gel (A), antigen–antibody reactions with the monoclonal dengue antibody MCA2277 (AbD Serotec) (B), the polyclonal mouse anti-dengue EDIII serotype 3 antibody (C), and the polyclonal mouse anti-dengue EDIII serotype 4 antibody (D) are represented. Note that MCA2277 detected EDIII serotypes 1 and 2 (B). The polyclonal mouse anti-dengue EDIII serotype 3 antibody (C), and the polyclonal mouse anti-dengue EDIII serotype 4 antibody (D) had strong cross reactivity with other serotypes. Lanes 1 to 5 contain 0.5 μg of *E. coli*-expressed serotype 1, 2, 3, 4 antigens, and scEDIII, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EDIII-Co1 and Tet-EDIII protein. When 3 μl of a 300-fold concentrated culture filtrate was resolved via SDS-PAGE, expected immunoreactive bands around 40 kDa were observed (Fig. 5D). These Western blot analyses using culture filtrates indicated that the produced Tet-EDIII-Co1 and Tet-EDIII proteins were successfully maturated to secret into the culture medium as expected (Fig. 5D).

ELISA using commercial antibodies was employed in order to conduct quantitative estimates of Tet-EDIII-Co1 and Tet-EDIII protein. The quantity of yeast-derived recombinant Tet-EDIII-Co1 and Tet-EDIII protein was estimated via the comparison of relative light units (RLU) from a known quantity of bacterial scEDIII antigen–antibody complex with that emitted from a known amount of yeast CFE and culture filtrate. When the recombinant protein levels were plotted against the CFE dilutions, a correlative increase in Tet-EDIII-Co1 protein levels was observed within the range of the CFE concentrations (0.2 μg–3.0 μg). However, when the CFE concentration deviated from these concentrations, the quantity of detected Tet-EDIII-Co1 protein decreased. This phenomenon can be ascribed to the binding characteristics of the target protein to microtiter plates in a mixture of total yeast protein. With increases in the total protein levels, the increasing amount of recombinant Tet-EDIII-Co1 may be unable to bind to the wells and eventually get lost through washing. Quantitative ELISA revealed that the yield comprised 0.1–0.2% of total soluble protein preparations as inferred from comparison with known amounts of scEDIII. Accordingly, the recombinant Tet-EDIII was also measured and it was estimated that the yield was 0.1–0.2% of total soluble protein. Quantitative ELISA showed that the maximum amount of Tet-EDIII-Co1 and Tet-EDIII proteins secreted into culture media was estimated to be 0.2 mg per liter of the culture filtrate for both recombinant proteins. The yields of recombinant Tet-EDIII-Co1 and Tet-EDIII were additionally estimated via side-by-side comparison of known concentrations of bacterial scEDIII preparation band intensity of with those of yeast-derived Tet-EDIII-Co1 and Tet-EDIII proteins in Western blot analysis (Fig. 5B and D). This quantitative Western blot analysis gave us estimated ranges from 0.1% to 0.2% of total soluble protein for CFE and 0.15–0.2 mg per liter for culture filtrates, respectively, which was in agreement with the results of quantitative ELISA analyses. Similar results using different antibodies were obtained (data not shown). Although the yield of target protein needs to be improved for further application, the calculated amount of target protein is likely to be underestimated considering the absence of reactivity to the serotype 3 and 4 antigens by the commercial monoclonal antibody MCA2277 and the variations of cross-reactivity of each antigen to the specific antibodies.

***In vivo* antigen binding assay of recombinant Tet-EDIII-Co1 and Tet-EDIII proteins** Since affinity for M cells is a preliminary requirement for an effective oral vaccine, an *in vivo* antigen uptake assay of Tet-EDIII-Co1 and Tet-EDIII was used to compare the relative binding of Tet-EDIII-Co1 and Tet-EDIII for M cells on PPs. M-cell specific targeting capability of the yeast-produced Tet-EDIII-Co1 and Tet-EDIII was measured relative to the

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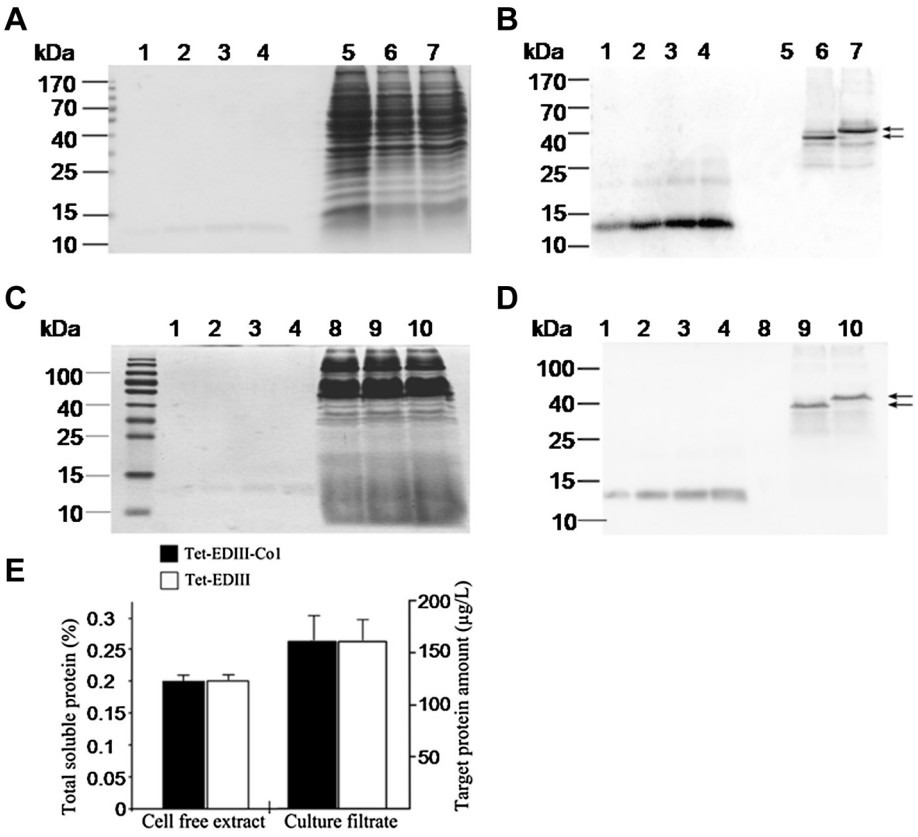

FIG. 5. Western blot and ELISA analysis of Tet-EDIII-Co1 and Tet-EDIII transformants. A Coomassie blue-stained SDS-PAGE gel of CFEs (A), its corresponding antigen–antibody reactions with the commercial monoclonal antibody MCA2277 (AbD Serotec) (B), a Coomassie blue-stained SDS-PAGE gel of concentrated culture filtrates (C), and its corresponding antigen–antibody reactions with the monoclonal antibody MCA2277 (D) are represented. Note that the presence of Tet-EDIII-Co1 and Tet-EDIII in CFEs (B) and culture filtrates (D) was confirmed with the monoclonal antibody MCA2277. Lanes 1 to 4 contain 20, 40, 60, and 80 ng of scEDIII, respectively. Lanes 5 to 7 contain 40 μg of total soluble protein from the mock, Tet-EDIII, and Tet-EDIII-Co1 transformants, respectively (A, B). Lanes 8 to 10 contain 3 μl of a 300-fold concentrated culture filtrate from the mock, Tet-EDIII, and Tet-EDIII-Co1 transformants, respectively (C, D). Arrows indicate Tet-EDIII and Tet-EDIII-Co1, which is slightly larger than Tet-EDIII. (E) The expression level of Tet-EDIII-Co1 (closed bar) and Tet-EDIII (open bar) in CFEs and culture filtrates from preparations of 3-day-old corresponding cultures are represented as a percentage and the amount per liter of culture, respectively. Bacterial scEDIII protein serial dilutions were used to create a standard curve to measure the expression level of target proteins.

well-defined characteristics of M cell binding with lectin UEA-1 but not WGA. The interaction with the M-cell specific antibody NKM 16-2-4 was assessed using CLSM software. As shown in Fig. 6, when Tet-EDIII-Co1 was administered, there were well-defined patches of overlap between sections stained with anti-dengue antibody (green), M-cell specific lectin (red), and anti-M cell antibody (purple) in the merged image. However, when Tet-EDIII alone was administered, no interaction with M cells was observed with images similar to the isotype controls. Furthermore, when Tet-EDIII-Co1 binding ability was examined with the M cell anti-complement receptor C5aR antibody, the binding of Tet-EDIII-Co1 overlapped with that of the C5aR receptor on the M cell surface. These results indicate that the Tet-EDIII-Co1 protein bound to M-cells, particularly to the C5aR receptor, on the M cell surface.

DISCUSSION

Even though yeast expression systems have several advantages over other eukaryotic expression systems, yeast systems have been historically avoided for the expression of human virus surface proteins (35). Several studies outlined the possible limitations of yeast expression systems to produce viral surface proteins including rather low expression levels (36,37), the formation of insoluble multimers (38,39), inactivity due to hyperglycosylation (40), and differences in molecular processes (35). Although we did not analyze every single protein fraction from our recombinant yeast system, the presence of tetrameric dengue virus EDIII in the soluble protein and culture filtrate suggested that the yeast-expressed human viral surface protein precursor matured appropriately in yeast cell for proper yeast secretion.

Discovery of the dengue EDIII as a neutralizing epitope (6–11) has opened up a great opportunity for the development of a safe and effective recombinant or DNA dengue vaccine based on this epitope (12,13,16,41–45). However, the requirements for a tetravalent EDIII vaccine are still inevitable. Our results showing the cross-reactivity of antibodies against *E. coli*-expressed EDIIIs of each serotype again suggested the difficulty and potential threat of antibody-dependent enhancement (46). Either a tetrameric tetravalent antigen created through fusion of EDIIIs from all four serotypes (12,14,15), or a single monomeric tetravalent antigen created by the development of a synthetic consensus protein (scEDIII) from the EDIIIs of all four serotypes (13,16) has been applied for the development of viable tetravalent subunit vaccine candidates. Recently, we expressed and confirmed the immunogenicity of scEDIII using *S. cerevisiae* (17) and plant (47) expression systems. In the current study, antigen–antibody reaction of yeast-expressed tetrameric EDIIIs to representative commercial antibodies indicated that the yeast-expressed and -matured tetramer maintained its epitope structures to interact with the commercial antibody, suggesting antigenic properties similar to the native viral protein. Compared to other expression systems, a balanced immune

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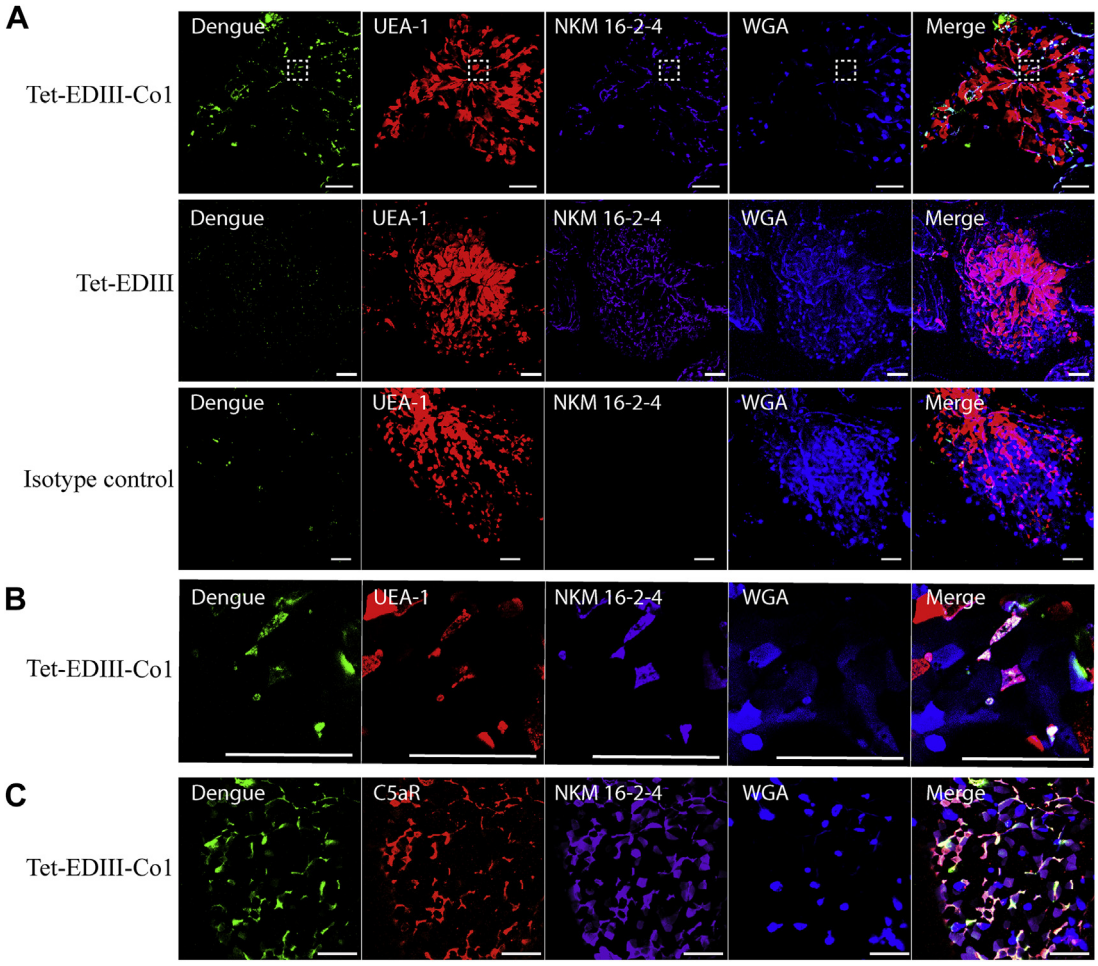

FIG. 6. *In vivo* antigen uptake assay. (A) CLSM images after staining Peyer's patch sections with anti-dengue (green), anti-M cell antibody (purple), UEA-1 (red) and WGA (blue). (B) Magnified CLSM images of inlets of the top panel of panel A. (C) Magnified CLSM images of Peyer's patch sections stained with anti-dengue, anti-M cell complement receptor antibody (C5aR, red), M cell-specific antibody (NKM 16-2-4), and WGA. Merged images are shown in the far right column. White bars represent 50 μm. Note that isotype control, commonly used in immunohistochemistry, is a negative control designed to measure the level of non-specific background signal caused by primary antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

response has been suggested for antigens produced in yeast expression systems (17). It will be of interest to compare the immunogenic efficacy of yeast-expressed tetrameric tetravalent or monomeric tetravalent epitopes.

Although tetravalent subunit EDIII vaccine candidates have achieved some success, the fact that they are all parenteral vaccines will pose difficulty for use in mass vaccinations, especially when the vaccines are targeted to affected populations in developing countries. The requirement of storage, formulation, and injections puts a great constraint on the already limited resources in these countries. Other issues involved in parenteral vaccine delivery such as contamination, pain, and anaphylactic shock will prevent compliance in patients (19). These problems will not be of concern with an oral dengue vaccine.

Due to our overall progress in understanding the mucosal immune system, particularly the gut immune system, various oral vaccine candidates have been developed against infectious diseases (18–20,47,48). Historically, most oral vaccine candidates were aimed at protecting against mucosal infections because it was thought that oral vaccines might not be well suited for non-mucosal infections such as dengue viral infection. This is not entirely true, as encouraging success has been achieved with the oral vaccination of non-mucosal infections such as malaria, Japanese encephalitis, and hepatitis B (21). Therefore, it is still possible to develop an effective oral dengue vaccine. Such vaccines could induce both mucosal as well as systemic immunity and may be easily administered without previous training.

The strategy for improving the efficacy of oral vaccines mainly involves supplementation with proper adjuvants. In the case of orally delivered vaccines, several adjuvants have been explored; among these are the toxin based *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT) as well as their non-toxic derivatives (19), the M-cell targeting monoclonal antibody (26), and the FimH (+) bacterial glycoprotein (25). While toxin-based adjuvants have been shown to be effective in inducing mucosal as well as systemic immunity in several studies, there is a size constraint for the fusion partner of the fusion protein for assembly into a functional conformation (49). On the other hand, the large size of the adjuvant in the case of monoclonal antibodies and glycoproteins may result in an unwanted immune response against these adjuvants.

M cells in the mucosal immune system are specialized antigen sampling epithelial cells and are easily accessible to microorganisms or migration molecules for tissue-specific consequences of lymphocyte priming in PPs (50,51). Thus, M-cell specific binding of orally administered antigens is essential for the simultaneous induction of effective mucosal and systemic immune responses. The recently discovered peptide ligand Co1 has been shown to target a fused antigen to M cell receptors and to induce an oral mucosal

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immune response (22). Compared to other surface markers for the potential delivery of mucosal vaccines through M cells, the Co1 ligand size is so small (12 amino acid residues) that it is easy to work with and it may not induce unwanted immune responses. Therefore, the current study demonstrating the M cell-specific targeting of the expressed tetrameric tetravalent antigen via the Co1 ligand indicated that this fusion antigen protein could be delivered to the specialized antigen sampling epithelial cells and successfully presented in antigen presenting cells of the mucosal immune system. These results are very promising for the development of a successful oral vaccine for the dengue virus infection. In addition, the successful use of a yeast expression system for tetrameric tetravalent antigens makes it possible to anticipate an easy preparation of an oral vaccine with a GRAS procedure and an additional enhanced immune response due to the strong adjuvant properties of the host derivatives.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2014.06.005.

ACKNOWLEDGMENTS

This work was supported by the Bio-industry Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, and in part by the NRF grants by MSIP (Nos. 2008-0061897 and NRF-2013R1A1A2012433). This research was also supported in part by the Korea Research Council of Fundamental Science & Technology (Joint Degree and Research Center for Biorefinery) and in part by "Cooperative Research Program for Agricultural Science & Technology Development" (Project no. PJ009999801), Rural Development Administration, Republic of Korea. We thank the Institute of Molecular Biology and Genetics at Chonbuk National University for kindly providing the facilities for this research. K. K. So was supported by BK21 PLUS program in the Department of Bioactive Material Sciences.

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