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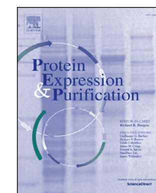

Expression and assembly of cholera toxin B subunit and domain III of dengue virus 2 envelope fusion protein in transgenic potatoes

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ABSTRACT

The rates of mosquito-transmitted dengue virus infection in humans have increased in tropical and subtropical areas. Domain III of dengue envelope protein (EDIII) is involved in cellular receptor binding and induces serotype-specific neutralizing antibodies. EDIII fused to the B subunit of *Vibrio cholera* (CTB–EDIII) was expressed in potatoes to develop a plant-based vaccine against dengue virus type 2. CTB–EDIII fused to an endoplasmic reticulum (ER) retention signal, SEKDEL, was introduced into potatoes by *A. tumefaciens*-mediated gene transformation. The integration of the CTB–EDIII fusion gene into the nuclear genome of transgenic plants was confirmed by genomic DNA polymerase chain reaction (PCR), and mRNA transcripts of CTB–EDIII were detected. CTB–EDIII fusion protein was expressed in potato tubers and assembled into a pentameric form capable of binding monosialotetrahexosylganglioside (GM1). The level of expression was determined to be ~0.005% of total soluble protein in potato tubers. These results suggest that dengue virus antigen could be produced in potatoes, raising the possibility that edible plants are employed in mucosal vaccines for protection against dengue infection.

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1. Introduction

Dengue is a common viral infection that is spread and transmitted to humans by *Aedes* mosquitoes. It is a global problem threatening half of the world's population [1], with 390 million cases of dengue infection estimated in 2010 [2]. Dengue is endemic in many tropical and sub-tropical regions with major outbreaks occurring in cycles every 3–5 years [3]. Dengue virus is a flavivirus, similar to Zika, Yellow fever, Japanese encephalitis and West Nile viruses. There is no specific treatment available for dengue.

Dengue virus has four antigenically distinct but related serotypes. These different serotypes are responsible for antibody-dependent enhancement of infection, which can induce severe symptoms such as fatal dengue hemorrhagic fever and dengue shock syndrome. A person infected with one of the four serotypes of dengue virus can develop severe dengue disease during a subsequent infection with a different serotype. The presence of non-neutralizing antibodies against a pre-existing serotype can enhance infection through binding of the antibody–virus complex to Fcγ receptors on circulating monocytes [4]. Thus, a dengue vaccine should induce sufficiently robust humoral and cellular immune responses including neutralizing antibodies to all four serotypes of virus.

Dengvaxia® vaccine developed by Sanofi Pasteur has been approved in Mexico, Philippines and Brazil; however, it shows only partial protection, with 60.8% overall efficacy against symptomatic dengue disease in children and adolescents 9–16 years old in phase 3 trials in Asia [5]. Importantly, the vaccine appeared to increase the risk of hospitalization among the very young (≥9 years old) in the third year of the follow up [6]. To meet the World Health Organization's aim of reducing dengue mortality by 50% and morbidity by 25% in 2020, it will be necessary to develop a dengue vaccine that is applicable to all ages and in all populations. Furthermore, such a vaccine should be easily accessible, affordable and show an excellent safety record.

Currently, the leading dengue vaccine studies have employed live attenuated virus vectors [7–9], with the envelope (E) protein of

Abbreviations: CTB, cholera toxin B subunit; E, envelope protein; EDIII, envelope protein domain III of dengue virus; ER, endoplasmic reticulum; PCR, polymerase chain reaction; TSP, total soluble protein; GM1, monosialotetrahexosylganglioside; MS, Murashige and Skoog; SDS, sodium dodecyl sulfate.

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dengue virus as the target antigen [10–12]. Domain III, the biologically critical portion of the E protein, has induced neutralizing antibodies in several studies utilizing various adjuvants [10,13,14]. Cholera toxin B subunit (CTB) has become an important adjuvant in developing oral vaccines owing to its ability to bind specific receptors (monosialotetrahexosylganglioside; GM1) on the intestinal surface and to carry vaccine-relevant antigens [15]. The nontoxic CTB component of cholera toxin naturally forms pentamers, and heterologous recombinant proteins can do the same. This feature is important in its binding to GM1, which is broadly distributed in various immune cells, including epithelial cells of the gut and antigen presenting cells, such as macrophages, dendritic cells and B cells. This binding leads to enhancement of the immune response to antigens, as demonstrated in some animal models [15].

Conservative vaccines consisting of inactivated or attenuated pathogens have safety and immunogenicity issues, including the risk of reverting to virulence, variability among species, low levels of immunogenicity and possible gene transfer to wild type strains [16,17]. Plant-based vaccines could potentially address some of these issues and help overcome the economic and safety limitations, especially in developing countries. Additionally, the vaccine antigens bioencapsulated in plant cells could be delivered efficiently to immune cells by oral administration, as was previously demonstrated in mice [18,19]. Many plant-based vaccine candidates have been explored to prevent human diseases such as influenza virus, tuberculosis, hepatitis B virus (HBV), human immunodeficiency virus, human papillomaviruses, rabies and malaria [20]. Edible plants such as tomato, lettuce, potato and carrot are considered attractive for vaccine production and oral delivery. Potato is an important food source in many parts of the world and has the potential for long-term storage, compared with, for instance, tomatoes or lettuce. Genetically modified potatoes could be used as a means for oral vaccine delivery. Such vaccines have been employed against certain pathogens such as *E. coli*, Norwalk viruses and HBV [21]; however, the lack of adjuvant requires further development.

In the present study, domain III of E protein (EDIII) of dengue virus serotype 2 was expressed in potatoes to develop a plant-based oral vaccine. To facilitate oral tolerance, CTB was fused to the EDIII antigen. We investigated the capacity of a transgenic potato to express the fusion protein CTB–EDIII. The genomic integration, mRNA expression and protein production of the target gene were demonstrated by genomic DNA PCR amplification, Northern blot and Western blot analyses, respectively. The binding capacity for GM1 was confirmed by GM1 enzyme-linked immunoassay (ELISA), showing that this expression system is suitable for production of biologically active CTB fusion proteins.

2. Materials and methods

2.1. Transformation and regeneration of plants

Previously, we described the plant expression vector pMYV498, containing cholera toxin B subunit and domain III of dengue virus E glycoprotein gene (CTB–EDIII) fused to an ER retention sequence (SEKDEL) [22]. In the present study, potato (*Solanum tuberosum* cv. Bintje) cells were transformed with *A. tumefaciens* LBA4404 carrying pMYV498 plasmid and PRK2013 helper plasmid to develop a plant-based vaccine against dengue infection. The plants induced from sterilized potato tubers were grown in Murashige and Skoog (MS) basal solid medium with 3% sucrose and 0.8% plant agar, pH 5.7 (MS), at 25 °C and then cut to collect internodes for *Agrobacterium*-mediated transformation. The prepared stems were incubated in *Agrobacterium* suspension solution for 15 min to allow penetration into the plant cells and then co-cultured with *A. tumefaciens* in MS basal medium in the dark for 2 days. To select transformed plant cells, the explants were transferred to selection medium [MS basic medium, 2 mg/L benzylaminopurine (BAP), 0.4 mg/L indole-3-acetic acid] containing antibiotics (100 mg/ml kanamycin, 300 mg/ml cefotaxime) and cultured for 4–5 weeks in the dark. For plant regeneration, the putative transformed calli induced from explants were transferred to shoot induction medium [MS basic medium, 2 mg/L BAP, 0.1 mg/L gibberellic acid (GA3), 100 mg/ml kanamycin, 300 mg/ml cefotaxime]. After incubation for an additional 3–6 weeks, the regenerated shoots from the calli were transferred to MS basal medium with antibiotics and without growth regulators to stimulate root formation.

2.2. Microtuberization of potato

To induce tubers, the potato plantlets (grown up to 6–8 cm) were transferred to tuber induction liquid medium (MS basal liquid medium containing 8% sucrose) and incubated under short-day treatment conditions (8 h) at 20–22 °C [23] (Fig. 1).

2.3. Detection of the CTB–EDIII gene in transgenic plants

To confirm the integration of the CTB–EDIII fusion gene into the potato genome, genomic DNA was isolated from wild type and putative transgenic potato leaf tissues using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The fusion gene was detected by PCR using CTB–EDIII specific primers (forward: 5′-GGT CTA GAG GAT CCG CCA CCA TGG TGA AG-3′ and reverse: 5′-GCG GTA CCT TTC TTG AAC CAG TTG AG-3′).

2.4. Detection of CTB–EDIII transcripts in transgenic plants

Total RNA was extracted from transgenic and wild type plants using Trizol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the supplier's instructions and concentrated by ethanol precipitation. Total RNA (30 μg) was separated by DNA electrophoresis on a 1.2% formaldehyde-agarose gel and then blotted onto a Hybond N^+ membrane (Amersham Lifescience Biotech, Buckinghamshire, UK). The membrane was hybridized with a ^{32}P-labeled CTB–EDIII probe using the Prime-a-Gene labeling system (Promega) at 65 °C in a hybridization incubator (FINEPCR Combi-H, Seoul, Korea). The blot was washed with 2× SSC buffer (300 mM NaCl, 30 mM Na-citrate) containing 0.1% and 1% sodium dodecyl sulfate (SDS) for 15 min at 65 °C, respectively, and then exposed to X-ray film (Kodak, Sevierville, TN, USA).

2.5. Detection of the CTB–EDIII protein in transgenic plants

Total soluble proteins (TSP) were extracted from non-transgenic and transgenic tuber tissues in 2 vol of 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) and centrifuged at 13,000 rpm for 15 min at 4 °C to remove insoluble cell debris. TSP was measured using the Bradford protein assay (Bio-Rad, Berkeley CA, USA), and 50 μg were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad) under non-reducing conditions, or 12% SDS-PAGE under reducing conditions, respectively. The gels were run at 120 V for 2–3 h in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, and 0.1% SDS). The recombinant CTB or EDIII proteins purified from *E. coli* were used as controls. The separated protein bands were transferred 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. To prevent non-specific antibody reactions, the membranes were

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