Expression of *Dengue* virus EIII domain-coding gene in maize as an edible vaccine candidate

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**Abstract**  Plant-based vaccines possess some advantages over other types of vaccine biotechnology such as safety, low cost of mass vaccination programs, and wider use of vaccines for medicine. This study was undertaken to develop the transgenic maize as edible vaccine candidates for humans. The immature embryos of HiII genotype were inoculated with *A. tumefaciens* strain C58C1 containing the binary vectors (V662 or V663). The vectors carrying *nptII* gene as selection marker and *scEDIII* (V662) or *wCTB-scEDIII* (V663) target gene, which code EIII proteins inhibit viral adsorption by cells. In total, 721 maize immature embryos were transformed and twenty-two putative transgenic plants were regenerated after 12 weeks selection regime. Of them, two- and six-plants were proved to be integrated with *scEDIII* and *wCTB-scEDIII* genes, respectively, by Southern blot analysis. However, only one plant (V662-29-3864) can express the gene of interest confirmed by Northern blot analysis. These results demonstrated that this plant could be used as a candidate source of the vaccine production.

**Keywords** Dengue virus, Edible vaccine, Immature embryo, Maize transformation, Paromomycin

**Introduction**

*Dengue* virus (DENV) is a mosquito-borne single positive-stranded RNA virus of genus *Flavivirus*. The genome of *Dengue* virus was 11,000 bases and codes three structural proteins of capsid protein C, membrane protein M and envelop protein E. The envelop protein E is on the viral surface and important for the initial attachment of the viral particle to the host cell with several molecules that interact with the viral E protein (Chang 1997; Seema and Jain 2005). The E protein contains three domains I-III and one trans-membrane domain. Domain III is the part in where the interaction between a receptor of a target cell surface and the virus occurs (Mukhopadhyay et al. 2005). Also the domain III possesses epitopes which elicit monoclonal antibodies, strictly inhibiting viral adsorption by cells (Crill and Roehrig 2001). Mice immunized by *Dengue* virus EIII protein produced from tobacco showed induced production of antibody against *Dengue* virus (Saejung et al. 2007).

Plant-based vaccines have some advantages such as low producing cost, safe storage, stable to protein denaturation and easy transportation. Some of commercial and medicinal proteins are produced from transgenic plants or cell suspensions. Several plant-derived oral vaccines have been reported, such as hepatitis B surface antigen (HBsAg) in tobacco and lettuce (Mason et al. 1992), *Norwalk* virus capsid protein in tobacco and potato tubers (Mason et al. 1996), LT-B (labile toxin B subunit) in potato tubers (Mason et al. 1998). However, the low expression level of target antigen in transgenic plants had been an obstacle to produce plant-based recombinant protein. Recently, the problem can be overcome by increasing antigen uptake into mucosal immune systems (Kim et al. 2010), and mucosal immune responses were increased by antigen uptake in gut epidermal cells (Saejung et al. 2007).

Corn is consumed worldwide as forage crops and cereals, and transgenic corn producing antigen protein will help...
the prevention of a disease more conveniently and lower the cost of vaccination. Particularly, transgenic grain seeds are not only appropriate systems for the oral delivery of subunit vaccines because of low water contents and long period of storage, but also attractive for the intensively genetic studies and the establishment of stable plant transformation system (Cho et al. 2005). Previously studies showed that Agrobacterium-mediated transformation in maize has been successfully (Kim et al. 2010), and especially transgenic maize as a candidate vaccine supply was also achieved (Shin et al. 2011).

In this study, we obtained transgenic maize plants expressing the DNA fragment of CTB (cholera toxin B subunit)-fused Dengue virus EIII domain using maize immature embryo transformation system to produce maize-derived antigen proteins.

Plant materials and methods

Plant materials

The maize seeds of Hi II genotype were obtained from the Maize Genetics Cooperation Stock Center (USDA, USA). For further use as plant materials of transformation, enough number of seeds were proliferated after pollination between A188 x B73 in greenhouse (Armstrong et al. 1991), and grown in greenhouse till maturity. The maize ears were harvested at 10 to 13 day after pollination, and stored up to three days at 4°C. Maize immature embryos (MIE) were aseptically dissected from the stored ears surface sterilized by 70% alcohol, and used for transformation according to Kim's methods (2009).

Preparation of A. tumefaciens solution

Agrobacterium tumefaciens strain C58Cl harboring the binary vectors carrying with scEDIII (V662) or with wCTB-scEDIII (V663), respectively, is used in this study. Both T-DNA of scEDIII (V662) and wCTB-scEDIII (V663) contain two cassettes (Fig. 1), one duplicated 35S Cauliflower Mosaic virus promoter-NPTII-NOS terminator as plant selection marker gene, and the other duplicated 35S Cauliflower Mosaic virus promoter-scEDIII gene (399bp)-NOS terminator for scEDIII (V662) or duplicated 35S Cauliflower Mosaic virus promoter-cholera toxin B subunit fused EDIII gene (729bp, wCTB-scEDIII)-NOS terminator for wCTB-scEDIII (V663) as target gene, respectively. A single colony of the strain was grown in YEP liquid medium containing 50 mg/l rifampicin, 50 mg/l gentamycin and 50 mg/l kanamycin, at 28°C in dark for 2 days. Then the pellet was harvested and suspended with AB low-phosphate liquid medium OD650 = 0.2, and cultured for 16 hours. Again the pellet was harvested and re-suspended with liquid co-cultivation medium for MIE transformation.

Maize immature embryo transformation

The harvested maize ear was sterilized twice with 70% ethanol for 10 min following Kim’s method (2009). After surface sterilization, a long forceps was plunged into the com cob vertically to make it easy to grab. The upper sides of kernel were cut off using a surgical blade (No. 11), then the 1.5 ~ 2.0 mm of immature embryos in size were isolated from each kernel by spatula and then were put into liquid co-cultivation medium to avoid dehydration. The MIEs were immersed in A. tumefaciens suspension for 5 min, and transferred onto solid co-cultivation medium at 28°C in dark for 2 days after removing the suspension by pipette. Then, the infected MIEs were cultured on delay medium to remove bacterial cells at 28°C in dark for 4-5 days. After delay culture, scutellum parts of embryos were excised from the germinated embryos and then cultured on selection medium with 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2, 4-D) and 50 mg/l paromomycin for the first two weeks. The scutellums were sub-cultured on same selection

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Fig. 1 T-DNA region of the binary vector V662 and V663; V662 carries scEDIII of 399 bp size fragment and V663 carries wCTB-scEDIII of 729 bp size of fragment and both carry NPTII gene for selectable marker.
medium containing 100 mg l⁻¹ paromomycin for 8 weeks totally in dark with a transfer every two weeks. Through 10 weeks selection, resistant calli were induced from the outer surface of scutellum, among which the callus proliferated from one scutellum was called a clone. The somatic embryos developed from the clones were transferred to the first regeneration medium for one week (25°C, light condition). Somatic embryos with greening were transferred onto second regeneration medium (25°C, light condition). After 15–20 days, regenerated plantlets (T0) in normal morphology were acclimated into soil and grown to maturity in greenhouse. The T0 plants were artificially self-pollinated or cross-pollinated with Hi II genotype, and the T1 seeds were harvested. All the media used were prepared by Kim’s methods (2009).

Southern and Northern blot analysis

For Southern blot analysis, genomic DNA was isolated from approximately 2 g of young leaves of putative transgenic maize plants (Dellaporta et al. 1983). About 50 μg DNA was digested with BamHI for 16 hours at 37°C, and then did electrophoresis on 0.8% agarose gel. The DNA in agarose gel was blotted onto Zeta-Probe nylon membrane (Bio-Rad, catalog #162-0196) in 20X SSC. The 750-bp nptII PCR product was labeled with 32P-dCTP using labeling mix (Amersham, catalog # RPN1633). The primer pairs for NPTII PCR were 5′-GAGGCTATTCGGCTATGACT-3′ and 5′-ATCGGGAGCGGCGATACCGT-3′.

For Northern blot analysis, total RNAs were isolated from the same plant samples for Southern blot analysis, using Tri-reagent (Molecular Research Center Inc., catalog # TR-118). Approximately, 15 μg of total RNAs from each sample was electrophoresised on 1% agarose gel containing 5.3% (v/v) formaldehyde, and then blotted onto Zeta-Probe nylon membrane (Bio-Rad, catalog #162-0196) in 20 X SSC. The PCR products for scEDIII gene (399 bp) and wCTB-scEDIII were labeled with 32P-dCTP using labeling mix (Amersham, catalog #RPN1633), respectively, for RNA-blot hybridization. The PCR products were amplified using primer sets of 5′-ATGATTAAATTAAAATTT-3′ as forward primer and 5′-AAGTTCATCCTTTTCGGA-3 as reverse primer) for scEDIII or wCTB-scEDIII gene.

Results and discussion

Generation of transgenic maize plants

To produce transgenic maize plants, eight experiments were performed, and 721 immature embryos in total were used for the experiments. After co-cultivation, the germinated embryos on the delay medium were removed, and then the scutellums were sub-cultured on the selection medium for four times at two weeks intervals to select callus clones. Among the infected immature embryos, 175 scutellum explants gave rise to callus clones and each clone was vigorous propagated on the selection medium. Somatic embryos induced from the clones were transferred onto 1st regeneration medium and then 2nd regeneration medium to grow the plantlets. All procedures carried out by Kim’s methods (2009). 22 regenerated plantlets with normal morphology were potted to vermicate and sealed with plastic bags to keep enough humidity. After one-week of acclimated, plastic bags were removed and grown in greenhouse until maturity (Fig. 2). In other wise, the average transformation frequency based on paromomycin resistance plantlets was 3.05%. The plantlets resistant to paromomycin were only 1 (1.49%), 2 (2.50%), 5 (6.02%) and 19 (19.0%) from the experiments of V662-25, V662-29, V663-32 and V663-33, respectively. Whereas, plants were not regenerated from V662-27, V662-33, V663-25 and V663-28 (Table 1). Since a stable and efficient of transformation using immature embryos (Cho et al. 2005) or type II callus (Kim et al. 2009) of maize had been reported in our
Table 1  Analysis of transgenic maize plants carrying with scEDIII or wCTB-scEDIII genes in molecular level

<table>
<thead>
<tr>
<th>Genes</th>
<th>Experiment No.</th>
<th>No. of immature embryos infected</th>
<th>No. of selected callus clones</th>
<th>No. of putative transgenic plants regenerated from the selection medium (%)</th>
<th>No. of transgenic plants confirmed by Southern blot analysis (%)</th>
<th>No. of expression of scEDIII gene in transgenic plant by Northern analysis</th>
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<tr>
<td>scEDIII</td>
<td>V662-25</td>
<td>67</td>
<td>19</td>
<td>1 (1.49)</td>
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<td>0</td>
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<td></td>
<td>V662-27</td>
<td>112</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>V662-29</td>
<td>80</td>
<td>19</td>
<td>2 (2.50)</td>
<td>2 (2.50)</td>
<td>(V662-29-3942)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>(V662-29-3864)</td>
</tr>
<tr>
<td>wCTB-scEDIII</td>
<td>V662-33</td>
<td>58</td>
<td>0</td>
<td>0 (0.00)</td>
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<td>0</td>
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<td></td>
<td>V663-25</td>
<td>104</td>
<td>13</td>
<td>0 (0.00)</td>
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<tr>
<td></td>
<td>V663-28</td>
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<td>17</td>
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<td></td>
<td>V663-32</td>
<td>83</td>
<td>11</td>
<td>5 (6.02)</td>
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<td></td>
<td>V663-33</td>
<td>96</td>
<td>55</td>
<td>12 (12.24)</td>
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<td>V663-29</td>
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<td>V663-33</td>
<td>721</td>
<td>175</td>
<td>22 (3.05)</td>
<td>8 (1.11)</td>
<td>1</td>
</tr>
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</table>

previous study, and transgenic maize expressing EPSP herbicide resistant gene (Cho et al. 2007) and Actinobacillus pleuropneumoniae ApxI A gene as vaccine candidate (Kim et al. 2010) were developed. By the stable and efficient protocols, putative transgenic plants based on paromomicin-resistance were produced, although the transformation frequency was lower than 5.5% (Frame et al. 2002) and higher than our previous study with 0.6% (Cho et al. 2005; Kim et al. 2009). In general, the factors influencing transformation efficiency include plant species, explants types, maturity, researcher skill, selectable marker, etc. (Gaba et al. 2004). In this study, the difference of transformation frequency from other studies may be speculated that the maturity of immature embryos was affected.

Southern blot and Northern blot analysis for transgenic maize plants

The integration of T-DNA was confirmed by Southern blot analysis (Fig. 3). Of twenty-two putative transgenic plants regenerates, eight plants were showed the integration of the target genes (scEDIII or wCTB-scEDIII) into the maize genome, which were labeled as V662-29-3864 and V662-29-3942 regenerates from V662-29 experiment (scEDIII gene), V663-33-3559 from V663-33 (wCTB-scEDIII), and V663-33-3326, V663-33-3783, V663-33-4034, V663-33-4094, V663-33-4098 from V663-33 (wCTB-scEDIII), respectively.

However, other fourteen putative transgenic plants were not verified for the integration of T-DNA region by Southern analysis. In other wise, total RNAs were isolated from the leaves of the putative transgenic plants. Of them, the expression of Dengue EDIII domain only showed in one plant (V662-29-3864), and the other seven plants had no expression, even though the Dengue EDIII gene successfully integrated into the genome (Table 1, Fig. 4). In transgenic

Fig. 4 Northern blot analyses of total RNA extracted from transgenic maize plants (T0 generation). The RNA (15 μg) was separated in 1% agarose gel in each lane and subjected to Northern hybridization. The 399bp scEDIII gene PCR product was labeled with [32P]dCTP and then used as probe. Negative control: non-transgenic plant, Transgenic plants: V663-33-4098, V663-33-3942, V663-32-3559, V663-33-3326, V663-33-3783, V663-33-4034, V663-33-4094

plants, the introduced target genes are sometimes silenced. An important factor of gene silence is generally known for the integrated location of transgene and the multi-copy per integration site (Stam et al. 1997). In special, expression of the transgene may be activated if becoming integrated into euchromatin of chromosome (Koncz et al. 1989), whereas inactivated if inserted into repetitive DNA or heterochromatin of chromosome (Pröls and Meyer 1992), and also often show low expression in case of multi-copy (Jones et al. 1987). Like these, non-expression of scEDIII gene in the seven plants may be speculated by gene silencing mechanism, except for the plant of V662-29-3864. Transgenic seeds (T1) were harvested after self- or cross-pollinated, dried and kept in a refrigerator in order to further immune response experiment in mice as subunit vaccine candidate. This result showed the possibility of maize as an appropriate plant-host for antigen production.

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