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Research Article

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

Hyun A Kim · Suk Yoon Kwon · Moon Sik Yang · Pil Son Choi

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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 inhibite 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 candidated source of the vaccine production.

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

H.A. Kim · S. Y. Kwon
Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea
Biosystems and Bioengineering Program, University of Science and Technology, Yuseong-gu, Daejeon, Republic of Korea

M. S. Yang
Division of Biological Science, Chonbuk National University, Jeonju Korea

P. S. Choi (✉)
Department of Medicinal Plant Resources, Nambu University, Gwangju 506-824, Republic of Korea
e-mail: cps6546@hanmail.net

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 overcame 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 (Amstrong 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 C58C1 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 mgl^{-1} rifampicin, 50 mgl^{-1} gentamycin and 50 mgl^{-1} kanamycin, at 28°C in dark for 2 days. Then the pellet was harvested and suspended with AB low-phosphate liquid medium OD_{650} = 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 corn 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 mgl^{-1} 2,4-dichlorophenoxy acetic acid (2, 4-D) and 50 mgl^{-1} paromomycin for the first two weeks. The scutellums were sub-cultured on same selection

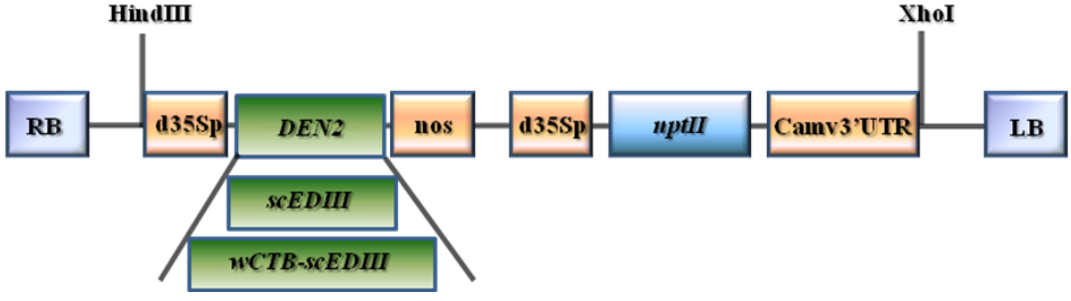

Fig. 1 T-DNA region of the binary vector V662 and V663; V662 carries *scEDIII* of 399 bp size fragment and V663 carries *wCTB-scEDIII* of 729 bp size of fragment and both carry *NPTII* gene for selectable marker

medium containing 100 mgl^{-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 (T_0) in normal morphology were acclimated into soil and grown to maturity in greenhouse. The T_0 plants were artificially self-pollinated or cross-pollinated with Hi II genotype, and the T_1 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 *Bam*HI for 16 hours at 37°C, and then did electrophoresis on 0.8% agarose gel. The DNA in agarose gel was blotted onto Zeta^R-Probe nylon membrane (Bio-Rad, catalog #162-0196) in 20X SSC. The 750-bp *nptII* PCR product was labeled with ^{32}P-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 ^{32}P-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 vermiculate 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

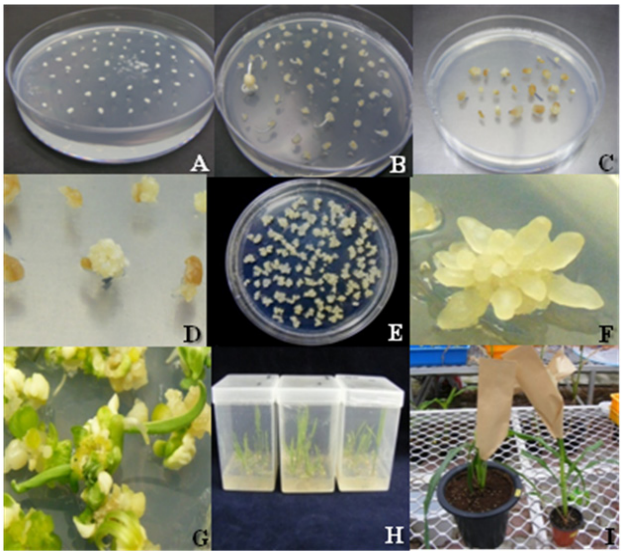

Fig. 2 Regeneration of transgenic maize plants from the cultures of immature embryos using *Agrobacterium*-mediated transformation method. A: Immature embryos on co-cultivation medium after infected with *Agrobacterium* solution. B: Immature embryos on delay medium. C: Immature embryos on selection medium containing 50 mgl^{-1} paromomycin. D: Callus induced from immature embryos inoculated. E: Propagation of callus on selection medium containing 100 mgl^{-1} paromomycin. F: Somatic embryos induced from callus clones. G: Germination of somatic embryos on 1st regeneration medium. H: Plantlets on germination medium. I: Fertilization of transgenic maize plants

Table 1 Analysis of transgenic maize plants carrying with *scEDIII* or *wCTB-scEDIII* genes in molecular level

Genes	Experiment No.	No. of immature embryos infected	No. of selected callus clones	No. of putative transgenic plants regenerated from the selection medium (%)	No. of transgenic plants confirmed by Southern blot analysis (%)	No. of expression of *scEDIII* gene in transgenic plant by Northern analysis
scEDIII	V662-25	67	19	1 (1.49)	0	0
	V662-27	112	0	0	0	0
	V662-29	80	19	2 (2.50)	2 (2.50) *(V662-29-3864)* *(V662-29-3942)*	1 *(V662-29-3864)*
	V662-33	58	0	0 (0.00)	0	0
wCTB-scEDIII	V663-25	104	13	0 (0.00)	0	0
	V663-28	80	17	0 (0.00)	0	0
	V663-32	83	11	5 (6.02)	1 (1.20) *(V663-32-3559)*	0
	V663-33	100	57	19 (19.00)	5 (5.00) *(V663-33-3326)* *(V663-33-3783)* *(V663-33-4034)* *(V663-33-4094)* *(V663-33-4098)*	0
Total		721	175	22 (3.05)	8 (1.11)	1

previous study, and transgenic maize expressing EPSP herbicide resistant gene (Cho et al. 2007) and *Actinobacillus pleuropneumoniae* ApxIIA gene as vaccine candidate (Kim et al. 2010) were developed. By the stable and efficient protocols, putative transgenic plants based on paromomycin-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 regenerated, 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 regenerated from V662-29 experiment *(scEDIII* gene*)*, V663-32-3559 from V663-32 *(wCTB-scEDIII)*, and V663-33-3326, V663-33-3783, V663-33-4034, V663-33-4094, V663-33-4098 from V663-33 *(wCTB-scEDIII)*, respectively.

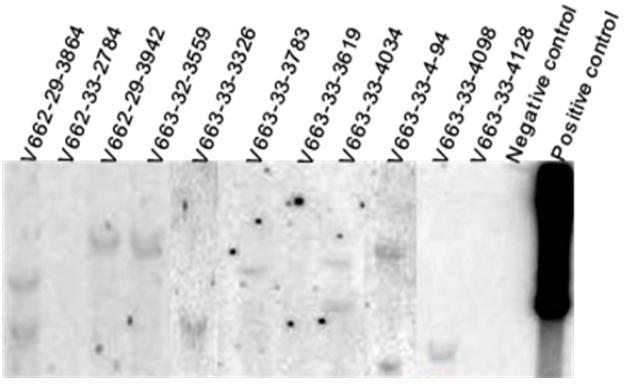

Fig. 3 Southern blot analysis of 11 putative transgenic cucumber carrying *nptII* gene. Genomic DNA was digested with *BamHI* and hybridized with 750bp *nptII* probe DNA labeled with ^{32}P-dCTP. Positive control: V662 vector, Negative control: Non-transgenic plant, Putative transgenic plants: V663-33-4128, V663-33-4098, V663-33-4094, V663-33-4034, V663-33-3619, V663-33-3783, V663-33-3326, V663-32-3559, V662-29-3942, V662-33-2784, V662-29-3864

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

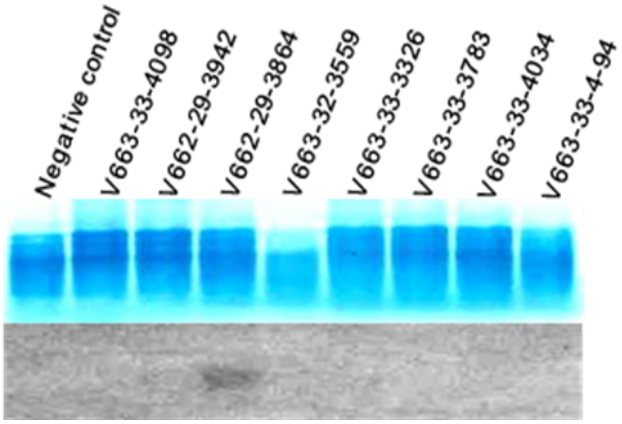

Fig. 4 Northern blot analyses of total RNA extracted from transgenic maize plants (T_0 generation). The RNA (15 μg) was separated in 1% agarose gel in each lane and subjected to Northern hybridization. The 399bp *scEDIII* gene PCR product was labeled with [32P]dCTP and then used as probe. Negative control: non-transgenic plant, Transgenic plants: V663-33-4098, V662-29-3942, V662-29-3864, 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 (T_1) 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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