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Expression of Porcine Epidemic Diarrhea Virus Spike Gene in Transgenic Carrot Plants

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ABSTRACT

This study was carried out to obtain basic information for possibility of oral vaccine in carrot using *Agrobacteruim* -mediated transformation system. The epitope region of porcine epidemic diarrhea virus (PEDV) spike gene which is classified as a member of the *Coronaviridae* and causes an acute enteritis in pigs was successfully expressed in carrot (*Daucus carota*) using the *Agrobacterium*-mediated transformation system. Hypocotyl segments of *in vitro* germinated plantlets were infected with *Agrobacteriun tumefaciens* LBA 4404 harboring PEDV spike gene. Embryogenic callus (EC) was induced on MS selection medium with 1 mg/L 2,4-D, 50 mg/L kanamycin and 300 mg/L cefotaxime after 45 days of culture. Subcultured ECs on MS selection medium without 2,4-D were converted to somatic embryos (SE) of various stage; globular, heart and torpedo stage. Putative transgenic embryos were selected on MS medium with 50 mg/L kanamycin and 300 mg/L cefotaxime. Regenerated plantlets from transformed SE were induced on MS medium containing 50 mg/L kanamycin after 30 days of culture. Genomic PCR confirmed the integration of PEDV spike gene into nuclear genome of carrot and northern blot analysis demonstrated the expression of PEDV spike gene in transgenic carrot.

Key words **:** PEDV, somatic embryogenesis, transgenic carrot

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is classified as a member of the *Coronaviridae* and causes an acute enteritis in pigs. The death rate of swine infected by PEDV is over than 95% and there is no effective vaccine for this disease. Up to now little was known about the PEDV gene and the expression of viral proteins. Recently the use of transgenic plants have been proposed as an alternative to produce and deliver vaccines (Stephen et al, 2001). Also, transgenic plants transformed with antigenic epitope were proposed are under development as edible vaccines. There are several reports demonstrating that antigens derived from various pathogens can be synthesized at high levels and in their authentic forms in plants (Arakawa et al., 1997; Gomez et al., 1998). Peter et al. (1995) reported the expression of gene for the glycoprotein (G-protein) which coats the outer surface of the rabies virus in tomato plants. The gene expression in-planta of PEDV

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spike has not reported before. In comparison with injected vaccine, oral vaccines have the advantage of being safe and easy to administer. Plant-based vaccine system, in particular, have several advantages; they are cheaper, have longer shelf-life and are safer (Lauterslager et al, 2001). We carried out the present study to express the PEDV spike gene and to investigate possibility of oral vaccine using *Agrobacterium*-mediated transformation system in carrot (*Daucus carota*) This basic research for integration of PEDV spike gene using carrot should provide the information for the development of edible oral vaccines.

MATERIALS AND METHODS

Expression vector construction and plant materials

The pBES plasmid containing PEDV spike gene was supplied by INRA (France). A pairs of primers (forward primer 5'-GAA TCT AGG ACA GCC, AAT TTC TAT GGT TAC TTT GCC TTT GCC ATC-3' and reverse primer 5'-CTG GAT CCT AAA TTA AAC GTC AAC TGT GAT ACC-3') were designed to generate the 460 bp PCR fragment containing epitope region of PEDV spike gene. The PCR products were cloned into pBlue Script KS vector to generate the pMYO38. The recombinant plasmid (PMYO38) was digested with *Xba* I and *Kpn* I and cloned into the same restriction site of the pMY27 plant expression vector. For the plant transformation, *Agrobacterium tumefaciens* strains LBA4404 was transformed into binary expression vector pMY27 by triparental mating using the *E. coli* strain HB101 containing pRK2013 as the helper. This vector carries the PEDV spike gene fused to a CaMV 35S promoter with a duplicated enhancer region including the Ω DNA sequence from the coat protein gene of tobacco mosaic virus, and to the nos terminator (Fig. 1).

The seeds of *Daucus carota* were first sterilized in 70% ethyl alcohol for 30 sec, then surface-sterilized in 2% sodium hypochlorite solution for 15 minutes. Finally, the seeds were rinsed three or four times in sterile distilled water. They were germinated on MS medium (Murashige and Skoog, 1962) without plant growth regulators at 25 ±1℃ in the dark. For transformation 7 day- old hypocotyls (6-9 mm in length) were used. All media were supplemented with 8 g/L agar and 30 g/L sucrose, adjusted to pH 5.8 prior to adding agar, autoclaved at 121℃ for 15 minutes and dispensed into petri-dishes.

Carrot transformation

Agrobacterium was grown in the dark at 28℃ overnight in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 mg/L kanamycin. For transformation of carrot, the carrot hypocotyls were precultured on MS medium with 1.0 mg/L 2,4-D. After 2 days of preculture, explants were soaked in the bacterial suspension for 15 minutes and blotted with sterile filter paper and cocultured for 2 days on MS medium with 1.0 mg/L 2,4-D. After cocultivation the explants were transferred to MS

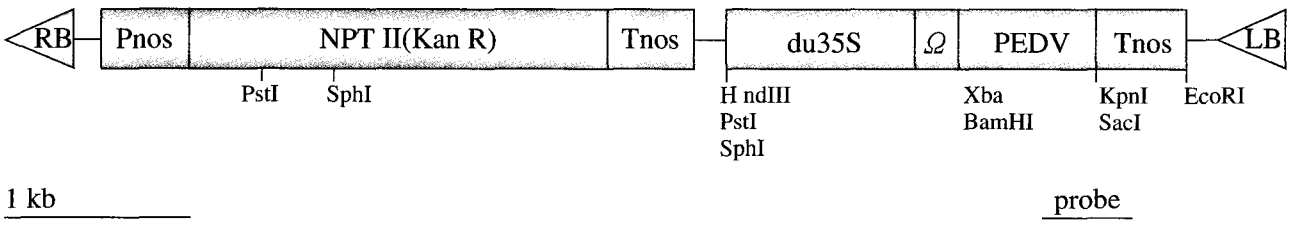

Fig. 1. Construction of plant expression vector containing PEDV spike gene

selection medium(SM) containing 1.0 mg/L 2,4-D, 50 mg/L kanamycin and 300 mg/L cefotaxime. The explants were transferred to fresh SM medium per 4 weeks. Putative transformed somatic embryos induced from embryogenic callus were transferred to MS medium without plant growth regulators and containing 50mg/L kanamycin and 300mg/L cefotaxime.

Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from young leaves of putative transgenic plants using the DNeasy plant mini kit (Qiagen) after grinding them with a mortar and pestle.

PCR analysis was accomplished using the primers (forward primer 5'-GAA TCT AGG ACA GCC AAT TTC TAT GGT TAC TTT GCC TTT GCC ATC-3' and reverse primer 5'-CTG GAT CCT AAA TTA AAC GTC AAC TGT GAT ACC-3'), which are specific for PEDV spike gene. PCR reaction was carried out with 1 unit Taq ploymerase, 2.5 mM dNTP mixture, 10 pM primer and 200 ng genomic DNA. The PCR was performed with 30 cycles, each consisting of 1 min at 94℃, 1 min at 55℃ and 1.5 min at 72℃. PCR products were electrophoresed on a 1.0% agarose gel and stained with ethidium bromide and examined on a uv transilluminator. Also, to identify PEDV specific gene we digested PCR products with restriction enzyme *Bgl* Ⅱ.

Northern blot analysis

Total RNAs were isolated from transgenic plants using the RNeasy plant total RNA mini kit (Qiagen). Total mRNAs were denatured and subjected to electrophores in a formaldehyde 1.2 % agarose gel and transferred to Hybond-N nylon membranes (Amersham) by capillary blotting according to the procedure of Sambrook et al.(1989). The RNA for northern blot analysis were hybridized with α-^{32}P-labeled probe with 0.46 kb fragment containing cDNA of PEDV spike gene.

RESULTS

Carrot tansformation

The main steps of somatic embryogenesis through transformation in carrot are shown in Figure 2. Embryogenic calli from the carrot hypocotyls were induced at the cut end of hypocotyls after 45 days of culture (Fig. 2A) and selected on the MS media containing antibiotics. After 6 weeks of culture, approximately 30 % of explants generated embryogenic callus at the cut end of explants but non-transformed explants did not form callus and they truned into brown color as culture period goes on. Putative transgenic calli were yellow or pale green and they showed vigorous growth on selection medium (Fig. 2B). Embryogenic calli transferred on MS medium with 50 mg/L kanamycin developed into various stages embryo: globular, heart and torpedo stage embryo (Fig. 2 C,D). Transformed somatic embryos were transferred to MS medium without plant growth regulators for complete plantlet development. The embryos of various stages developed not only normal with two cotyledons (Fig. 2E) but also abnormal plantlets (data not shown). Approximately 30% of the induced embryos were abnormal and they were turned to brown color on selection medium. Finally ten normal regenerated plants were selected and maintained in vitro for further analyses (Fig. 2F).

Genomic PCR and northern blot analysis

Genomic DNA was isolated from individual transgenic carrot plants. The presence of the PEDV spike gene in the transgenic carrot was analyzed using PCR with primers designed to amplify genomic DNA prepared from the transgenic carrot. The PCR products were electrophoresed on 1% agar gel. A single major band appeared at about 460 bp from the transgenic

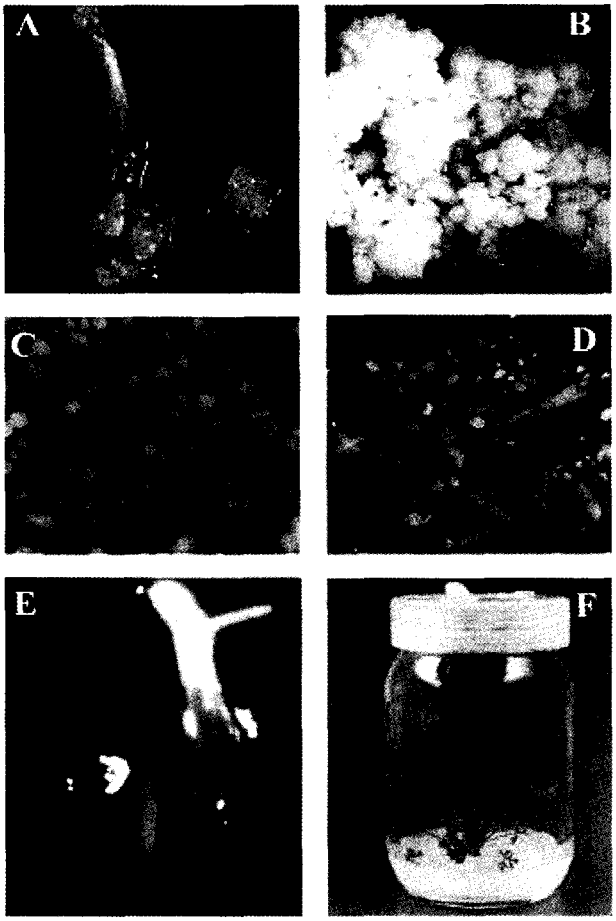

Fig. 2. Production of carrot (*Daucus carota*) transformed with PEDV gene.
A: Embryogenic callus (EC) formation on edge of explant on MS medium with 1 mg/L 2,4-D, 50 mg/L kanamycin and 300mg/L cefotaxime B: Vigorous ECs after transfer to fresh selection medium C: SEs of globular and heart stage on formed on MS selection medium without 2,4-D D: Torpedo stage embryos E: Germination of SE on MS selection medium without 2,4-D F: Normal plantlet transformed with PEDV gene on MS medium containing 50 mg/L kanamycin

carrot while there was no band observed from the negative control (non-transgenic plant) (Fig. 3). Also, PCR products were digested with *Bgl* II to identify PEDV specific DNA. As a result, two bands were appeared at site of 320 bp and 140 bp (Fig. 4). It suggested that PEDV spike gene was stably integrated into *Daucus carota* genomic DNA. Total RNA from leaves of PCR-positive PEDV transformants was used

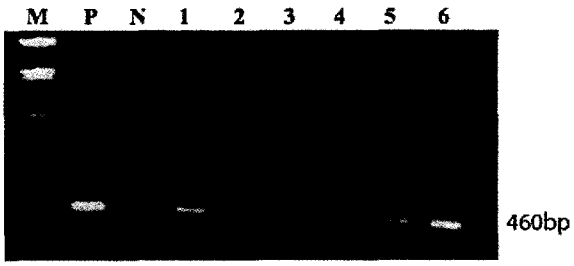

Fig. 3. PCR analysis from putative transgenic plants.
PC: Positive control(plasmid); NC: Negative control (non-transgenic plant); lane: 1 -6 transgenic carrot plants

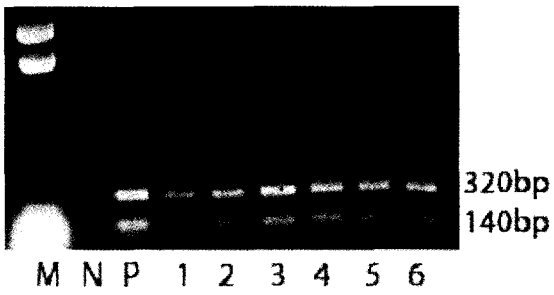

Fig. 4. Patterns of restriction enzyme reaction of PCR products digested with *Bgl II*
M: Size marker; N: Negative control (non-transgenic plant);
P: Positive control (plasmid); lane: 1 -6 transgenic carrot plants

for RNA- PEDV blot analysis. An α-^{32}P-labeled DNA probe when hybridized to RNA revealed a strong hybridizing signal. As expected, no amplification and hybridization was found in the samples prepared from non-transgenic plant (control plant). Four of the transformed plants expressed relatively high levels of PEDV transcripts (Fig. 4). These results suggested that introduced PEDV spike gene was successfully transcribed in the transgenic plants.

DISCUSSION

Plants are recognized as safe and cheap production system for proteins of pharmaceutical interest including vaccines (Tacket and Mason, 1995). Up to now transgenic plants expressing antigens of antibodies have

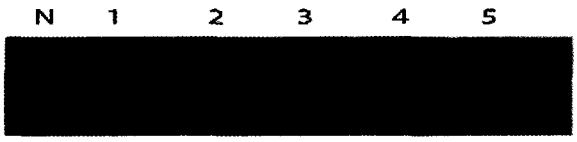

Fig. 5. Northern blot analysis from transgenic plants indicating the expression of PEDV spike gene N: Negative control (non-transgenic plants); lane 1 -5 transgenic plants

developed successfully using *Agrobacterium*-mediated transformation system (Mason and Arntzen, 1995; Ma and Vine, 1999). But the gene expression of PEDV which causes an acute enteritis in pigs was not reported in plant. The use of transgenic plants for vaccine production has several potential benefits over traditional methods. Firstly, transgenic plants are engineered to express only a small antigenic portion of pathogen, eliminating the possibility of infection and reducing the potential for adverse reaction. Secondly, since there are no known human or animal pathogens that are able to infect plants, concerns with viral or prion contamination are eliminated. Thirdly, direct oral administration is possible when the chosen proteins are expressed in commonly consumed food plants to give edible vaccines (Stephen et al, 2001). The carrot (*Daucus carota*), a member of the family *Umbelliferae*, is an important horticultural crop that is grown worldwide for its edible root, which contains high levels of vitamin A and other nutrient (Chen and Punja, 2002). We are exploring the use of carrot root as a particularly convenient delivery system for edible vaccines for human and animal. The normal use of plants as human foods and as animal feed, with the production of vaccine subunit components in plant tissues, should allow vaccines to be produced at a fraction of the cost of other approaches. The objective of this study was to introduce a PEDV spike gene and express them from transgenic carrot (*Daucus carota*) using *Agrobacterium*-mediated transformation system and to investigate whether transgenic carrots will have the possibility of vaccine or not. So, we tried to transform of carrot with PEDV gene and demonstrated the expression of PEDV gene using northern blotting analysis. These transgenic carrots using *Agrobacterium*-mediated transformation system will provide a valuable tool for the development of edible oral vaccines.

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