Oral immunization with rotavirus VP7-CTB fusion expressed in transgenic *Arabidopsis thaliana* induces antigen-specific IgA and IgG and passive protection in mice

YUXIAN LI[1-3], LILI GUAN[1], XIUMING LIU[1], WEICAN LIU[1], JING YANG[1], XIAOMEI ZHANG[1], FAWEI WANG[1], YONGXIN GUO[1], HAIYAN LI[1] and XIAOKUN LI[1,4]

[1]Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University, Changchun, Jilin 130118; [2]College of Traditional Chinese Medicine, Jilin Agricultural University, Changchun, Jilin 130118; [3]Traditional Chinese Medicine Department, Jilin Agricultural Science and Technology College, Jilin 132101; [4]College of Life and Environmental Science, Wenzhou University, Wenzhou, Zhejiang 325035, P.R. China

Abstract. Human rotavirus (HRV) is the primary cause of severe gastroenteritis in children. However, there is currently no protective virus for rotavirus available. In the present study, an HRVVP7-cholera toxin B subunit (CTB) fusion protein was expressed in *Arabidopsis thaliana*. To determine the adjuvant effect of HRVVP7-CTB, HRVVP7 without CTB was expressed in the same manner. HRVVP7-CTB accounted for 0.39% of the total soluble protein (TSP) in the transgenic seeds and 52.65 μg/g of HRVVP7 protein was expressed in these seeds. Mice were immunized with TSP from the transformed seeds and produced serum immunoglobulin G (IgG) and mucosal IgA specifically directed against HRVVP7. Antibody titers were highest in mice orally immunized with the plant-expressed HRVVP7-CTB protein, whereas HRVVP7-CTB-specific IgG neutralized the rotavirus. Suckling pups born from dams immunized with the HRVVP7-CTB fusion protein were protected against challenge with virulent rotavirus. The results of the present study suggest that the HRVVP7-CTB fusion protein produced in *A. thaliana* may be a rotaviral-specific candidate subunit vaccine.

Introduction

Human rotavirus (HRV) is the most frequent cause of viral gastroenteritis in young children, with 440,000 estimated deaths annually worldwide among children <5 years old (1,2). Rotaviruses are also able to infect other animals, including birds and companion and livestock animal species, causing extensive economic losses (3). Therefore, safe and effective vaccines against rotaviral infection are urgently required. At present, two new live oral attenuated rotaviral vaccines, Rotarix® (GlaxoSmithKline, Brentford, England) and Rotateq® (Merck KGaA, Darmstadt, Germany), have been approved for distribution. However, both are expensive and require strict conditions for their production and storage, limiting their use in developing countries (4,5). No protective subunit rotavirus vaccine is currently available. Rotavirus subunit vaccines raise neutralizing antibodies against the viral outer capsid protein VP7, which contains neutralizing epitopes that prevent viral attachment to the host cell membrane (6). A previous study demonstrated that different vectors that express HRV glycoprotein VP7 are able to stimulate protective immunity against infection and serve a key role in protection (7). According to an amino acid (aa) sequence analysis of rotavirus SA11, the VP7 protein has three major antigenic sites; A (aa 87-99), B (aa 145-150) and C (aa 211-223), which contain the virus neutralization epitopes (8). VP7 is also the dominant target protein for rotavirus-specific cytotoxic T-lymphocyte activity (9). VP7 was therefore selected as the best candidate protein for subunit vaccine development.

The use of plants as bioreactors for the production of foreign proteins has been increasingly directed towards the generation of experimental immunogens (10). Plants are a potentially inexpensive source of antigens that can be administered parentally or used as edible vaccines (11). Oral and nasal administration of plant-produced vaccines has elicited mucosal and systemic immune responses in animal and human trials (12). To date, numerous viral antigens that are immunogenic in animals have been produced in transgenic plants (13-18). Several other groups have studied rotavirus expression in plants, including VP7-RTB fusion (19), a human rotavirus candidate vaccine expressed in *Nicotiana benthamiana* (20). However, because proteins are digested in the intestinal tract, vaccination via the oral route may result in a significant reduction in

Correspondence to: Professor Haiyan Li or Professor Xiaokun Li, Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University, Building No. B1, 2888 Xincheng Street, Changchun, Jilin 130118, P.R. China
E-mail: hyli99@163.com
E-mail: proflxk@163.com

the amount of antigen delivered to antigen-presenting cells (APCs) in gut-associated lymphoid tissues (21,22). Increasing the production of antigenic proteins in transformed plants is often problematic; however, increasing the immunogenicity of plant-produced antigens may significantly enhance the mucosal immune responses (23).

Cholera toxin B subunit (CTB) is the nontoxic portion of the cholera toxin molecule that binds to the GM1 ganglioside receptor, a glycolipid expressed on most cells in the human body, including immune cells (24). Coupling an antigen to CTB increases the immunogenicity of the antigen as it increases receptor-mediated uptake and subsequent presentation of the antigen by APCs (25).

Based on these considerations, two plant expression vectors containing the nucleotide sequence of HRVVP7 or HRVVP7 linked upstream from the CTB subunit sequence were constructed to test the feasibility of expressing HRVVP7 and an HRVVP7-CTB fusion protein in transgenic *Arabidopsis thaliana*. Immunization with extracts of transgenic *Arabidopsis* seeds induced an efficient immune response in mice, which provided passive protection against infection with rotavirus SA11 in neonatal mice born to immunized dams. The immunogenic effects of HRVVP7 and HRVVP7-CTB were compared, with the aim of developing an adjuvant anti-rotavirus mucosal subunit vaccine.

Materials and methods

Construction of pPHAP1301-HRVVP7-linker-CTB and pPHAP1301-HRVVP7 expression vectors. The T-DNA region of the pPHAP1301 plasmid vector (supplied by the Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun, China) contains the phaseolin promoter, phaseolin terminator, 35S promoter, *bar* gene and the nopaline synthase gene *nos*. The HRVVP7 and CTB coding sequences were retrieved from a patent (patent no.: US 7,285,280B1) and GenBank (https://www.ncbi.nlm.nih.gov/nuccore/847821/), respectively and modified by codon optimization based on the codon usage of plants by Genewiz, Inc. (Suzhou, China). A 15-residue linker coding sequence $(Gly_4Ser)_3$ was used (26). Restriction sites *NcoI* and *HindIII* were introduced at the 5' and 3' ends of the coding sequence, respectively.

All these genes were then concatenated in the sequence HRVVP7-linker-CTB by Genewiz, Inc. The pPHAP1301 plasmid was digested with *NcoI* and *HindIII*. The HRVVP7 linker CTB gene was extracted from PUC19-HRVVP7-linker-CTB (Genewiz, Inc.) and digested with *NcoI* and *HindIII*. This HRVVP7-linker-CTB fragment was inserted into the cleaved pPHAP1301 plasmid by incubating them with T4 DNA ligase at 4°C for 10 h. The HRVVP7 sequence was subsequently amplified from the plasmid pUC19-HRVVP7-linker-CTB (Genewiz, Inc.), using polymerase chain reaction (2xTransTaq High Fidelity PCR SuperMix kit; TransGen, Inc., Beijing, China) with the following primers: Forward, 5'-CATGCCATGGGATAC ATGCCAGAACTCATG-3' and reverse, 5'-CCCAAGCTT AACTCTATAGTAgAAAGCAGC-3'. The thermal profile of the PCR program was: Initial denaturation at 94°C for 15 min; 35 cycles of 92°C for 30 sec, 52°C for 30 sec and 72°C

for 1 min; and a final extension at 72°C for 10 min. The PCR product was cloned into the pPHAP1301 expression vector to construct the recombinant plasmid pPHAP1301-HRVVP7. The expression vectors pPHAP1301-HRVVP7-linker-CTB and pPHAP1301-HRVVP7 were confirmed by PCR and a restriction enzyme analysis using the aforementioned protocol. The freeze-thaw method was used to transfer plasmids pPHAP1301-HRVVP7-linker-CTB and pPHAP1301-HRVVP7 into *Agrobacterium tumefaciens* EHA105 (cat. no. AC1010; Shanghai Weidi Bechnology Co. Ltd., Shanghai, China), and transformation was confirmed by PCR using the aforementioned protocol.

Transformation and selection of A. thaliana. *A. thaliana* ecotype Columbia specimens were selected for floral dipping when a pot of healthy plants (Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University) contained approximately 25 inflorescences and some maturing siliques. The siliques are routinely clipped off in the laboratory (27). The floral-dipping liquid medium contained 100 g/l sucrose, 1% B5 basal medium (AmyJet Scientific, Inc., Wuhan, China), 2 mg/l 6-benzylaminopurine, 1 M sodium hydroxide and 0.04% surfactant Silwet L-77 (GE Healthcare Life Sciences, Little Chalfont, UK) prepared as previously described (28). The plants were inverted and their aerial parts were dipped in *Agrobacterium tumefaciens* EHA105-containing floral-dipping medium for 7 min and wrapped in plastic film to maintain high humidity at 24°C for 20 h. The plastic covers were removed and the plants were grown at 24°C with 40% humidity conditions in a growth chamber until they were dried and their seeds (T1) were harvested with a sample bag.

The T1 seeds were grown in sterilized soil until the majority of the plants had produced six leaves per plant. The primary transformants were selected using 1% glufosinate (Sigma-Aldrich; Merck KGaA), which was sprayed onto the plants three times every 2 days. The cotyledons of the untransformed plants became chlorotic and bleached within 3-5 days, whereas the resistant seedlings grew healthy green leaves. The selected, glufosinate-resistant lines were demonstrated to contain the pPHAP1301-HRVVP7-linker-CTB or pPHAP1301-HRVVP7 sequence using PCR with the primers and conditions described above. A large number of homozygous T3 seeds were obtained with further rounds of plant reproduction. These T3 seed lines were used for a protein expression analysis and activity assays.

Total soluble protein (TSP) extract for oral immunization or immunoassay. The T3 seeds of transgenic *A. thaliana* were triturated in liquid nitrogen and mixed with protein isolation buffer [50 mM/l Tris-Cl (pH 8.0), 10 mmol/l EDTA, 100 mmol/l NaCl, 0.5% Triton X-100, 14 mmol/l mercaptoethanol and 1 mmol/l PMSF]. The mixture was centrifuged at 13,000 x g at 4°C for 22 min. The TSP content in the supernatant was determined using a Bradford assay (29). The final TSP samples were stored at -80°C for subsequent oral immunization or immunoassay.

Total RNA extraction and reverse transcription (RT)-PCR analysis of transformed A. thaliana seeds. Total RNA was extracted from the seeds of the transformed and wild-type

A. thaliana plants using a plant RNA extraction kit (MiniBEST Plant RNA Extraction kit; cat. no. 9769; Takara Bio, Inc.). The expressed pPHAP1301-HRVVP7-linker-CTB and pPHAP1301-HRVVP7 mRNA was amplified using an RT-PCR kit (PrimeScript™; cat. no. RR041A; Takara Bio, Inc.) with the following primers: Forward, 5'-CATGCCATGGGA TACATGCCAGAACTCATG-3' and reverse, 5'-CCCAAG CTTAGTTAGCCATAGAGATAGCAGCG-3'; and forward, 5'-CATGCCATGGGATACATGCCAGAACTCATG-3' and reverse, 5'-CCCAAGCTTAAACTCTATAGTAGAAAG CAGC-3', respectively. The PCR products were separated by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide at 25˚C for 18 min and visualized under UV light.

Western blotting analysis and ELISA quantification of proteins expressed in A. thaliana seeds. TSP (20 μg) from the transgenic and wild type seeds was separated by 10% SDS-PAGE. The resolved proteins were subsequently transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk at 25˚C for 24 h, followed by incubation with rabbit polyclonal antibody directed against rotavirus VP7 (cat. no. LS-C370878; LifeSpan BioSciences, Seattle, WA, USA; 1:1,000 dilution) at 25˚C for 2 h. Following three washes with TBST (15 min each wash), alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (Ig)G antibody (cat. no. ab98505; Abcam, Cambridge, UK; 1:10,000 dilution) was added and incubated at 25˚C for 2 h. Membranes were washed and antibodies were detected with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. PageRuler Plus Prestained Protein Ladder (cat. no. 26619 SM1811; Thermon Group Holdings, Sam Marcos, TX, USA) was used according to the manufacturer's protocol.

To quantify the amount of HRVVP7 expressed, 10 μl of TSP was diluted in 190 μl of 50 mM carbonate buffer (pH 9.6). A total of 50 μl/well of each diluted sample was used to coat a high-binding 96-well plate (Corning Incorporated, Corning, NY USA). Samples were blocked with 3% (w/v) skimmed milk at 25˚C for 1 h, followed by incubation with the rabbit polyclonal directed against rotavirus VP7 (1:1,000 dilution) at 25˚C for 1 h. Following three washes with TBST, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (cat. no. ab6721; Abcam; 1:20,000 dilution) was added and incubated at 25˚C for 1 h. Tetramethylbenzidine (TMB; 100 μl) was added for 10-15 min at room temperature, followed by 50 μl 2 M H_2SO_4 to stop the reaction. The absorbance was measured at 490 nm using a microplate reader. Aliquots of samples containing serially diluted rotavirus VP7 protein (cat. no. 80-1391; Fitzgerald Industries International, Inc., Concord, MA, USA) were used to construct a standard curve and the concentrations of VP7 in the samples were interpolated to determine the quantities of HRVVP7.

Oral immunization and antibody assessment. A total of 30 female 4-6 weeks old inbred BALB/c mice (18-20 g) were obtained from the Experimental Animal Center of Jilin Agricultural University. Mice were acclimatized to a 12 h light/dark cycle at 22±2˚C for 2 weeks with unlimited food and water in a specific pathogen-free facility. All animal experiments were approved by the Ethics Committee of the Jilin Agricultural University Institutional Review Board and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (30). Mice were divided into the HRVVP7-linker-CTB, HRVVP7 and negative control groups (10 mice/group). The groups were gavaged on days 0, 7, 14 and 21 with TSP from wild-type or T3 transgenic *A. thaliana* seeds.

Mice in the negative control group were gavaged with 10 mg of TSP from wild type *A. thaliana* seeds and 0.01 mg CTB adjuvant. Mice in the HRVVP7 group were gavaged with 8.3 mg of HRVVP7-containing TSP from transgenic *A. thaliana* seeds, which contained 25 μg of HRVVP7 protein from the transgenic seeds and 0.01 mg CTB adjuvant. Mice in the HRVVP7-linker-CTB group were gavaged with 6.2 mg of HRVVP7-linker-CTB-containing TSP from the transgenic *A. thaliana* seeds, which contained 25 μg of HRVVP7 protein from the transgenic seeds. Saliva and feces samples (homogenized in 0.01 M PBS) were collected weekly from for anti-HRVVP7 IgA analysis. On day 39, serum samples were collected and tested for their anti-HRVVP7 IgG titers. On day 53, 4 mice in each group were anesthetized with 2% isoflurane (Sigma-Aldrich; Merck KGaA) in 98% oxygen and euthanized by cervical dislocation. Mouse small intestines were subsequently collected and homogenized in 0.01 M PBS for the analysis of mucosal anti-HRVVP7 IgA. All antibody titers were evaluated with an indirect ELISA as described previously (13). In brief, a known concentration of rotavirus VP7 protein (Fitzgerald Industries International, Inc.) was probed with mouse serum, saliva, homogenized feces, or homogenized small intestine and detected using an HRP-conjugated goat anti-mouse IgG antibody (cat. no. HS201-01; TransGen Biotech, Beijing, China; 1:15,000 dilution) at 25˚C for 1 h. TMB (100 μl) was added at 37˚C for 10-15 min, followed by 50 μl of 2 M H_2SO_4 to stop the reaction. The absorbance was measured at 490 nm. Aliquots of samples containing the serially diluted rabbit polyclonal antibody directed against rotavirus VP7 (LifeSpan BioSciences) were used to plot a standard curve and the values for the samples were interpolated to determine the titers of the serum antibodies. The antibody titers were determined as the reciprocal of the highest antibody dilution yielding an absorbance at 490 nm (A_{490}) that was at least two-fold higher than the mean A_{490} for antibody samples from the negative controls. The results are presented as the mean ± standard deviation.

Neutralization assay. To determine the neutralizing activity of anti-HRVVP7 antibodies in the serum samples, the best result (as described above) was mixed with 100 μl 200 PFU of SA11 virus (provided by Dr. Zhen Lang Sun, Central Hospital of Fengxian District of Shanghai, Shanghai, China) in a 1:1 volumetric ratio. Neutralization assays were performed in MA104 cells [originally thought to originate from Rhesus macaque and later corrected to originate from African green monkey (31)], which are suitable for rotavirus research (32,33). The neutralized virus was subsequently added to the culture medium of MA104 cells and cultured at 37˚C in an atmosphere containing 5% CO_2. Following 2 h of culture, MA104 cells were cultured in fresh viral culture medium under same conditions for 18 h. The cells were then fixed with methanol at 25˚C for 30 min. The cells were incubated with 1% bovine

serum albumin and 22.52 mg/ml glycine (both Sigma-Aldrich; Merck KGaA) in PBS+0.1% Tween20 at 25°C for 30 min to block unspecific binding of the antibodies. The intracellular rotavirus was detected using the mouse mAb directed against rotavirus VP7 (cat. no. C01715M; Meridian Life Science, Inc., Memphis, TN, USA; 1:500 dilution) as the primary antibody at 25°C for 30 min and fluorescein-isothiocyanate-labeled goat anti-mouse IgG antibody (cat. no. ab6785; Abcam) as the secondary antibody (1:500 dilution) at 37°C for 1 h. The cells were washed three times with PBS at room temperature (15 min/wash). In the present study, wells containing no antibody were processed in parallel with the samples as negative controls. Results were assessed using a fluorescence microscope (Nikon E800; Nikon Corporation, Tokyo, Japan), at a magnification of x125. Neutralization was deemed to have occurred when the number of fluorescently labeled infected cells was only 60% of the number in the control wells.

Challenge experiment. The challenge experiment was based on the analysis of antibodies raised against HRVVP7-CTB and the HRVVP7 without CTB fusion protein. A total of 3 normal males inbred BALB/c mice (4-6 weeks old, 18-20 g) were obtained from the Experimental Animal Center of Jilin Agricultural University. Mice were acclimatized to a 12 h light/dark cycle at 22±2°C for 2 weeks with unlimited food and water in a specific pathogen-free facility. Two female mice from each group that showed the best immune response were mated with normal males and mouse pups were born after a 19-20 day gestation period. On day 5 post-parturition, 6 pups in each group were challenged with rotaviral strain SA11. Pups bred from the control mice gavaged with nontransgenic Arabidopsis seeds were used as the negative controls (n=6). The pups were challenged using a method previously described by Yu and Langridge (17). Each pup was challenged with 200 μl (100 PFU) of simian rotavirus strain SA11. Mice were weighed at 24-h intervals and the anus of each pup was checked three times a day. The mice from the infected group without watery feces were classified as 'non diarrheic' mice and the others were classed as 'diarrheic' mice. The intensity of diarrhea was determined according to the color of the anus and the amount of adherent feces. The number of pups showing diarrhea symptoms and the intensity of their diarrhea symptoms were observed for at least 10 days after challenge.

Statistical analysis. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance with Bonferroni's post-test correction was used to compare the differences between groups. $P<0.001$ was considered to indicate a statistically significant difference.

Results

Genetic and immunoblotting analyses of HRVVP7-linker-CTB and HRVVP7 from transformed A. thaliana seeds. Expression vectors carrying the HRVVP7-linker-CTB or HRVVP7 sequence were constructed to produce the HRVVP7-linker-CTB or HRVVP7 protein, respectively (Fig. 1). *A. thaliana* was transformed with the pPHAP1301-HRVVP7-CTB or pPHAP1301-HRVVP7
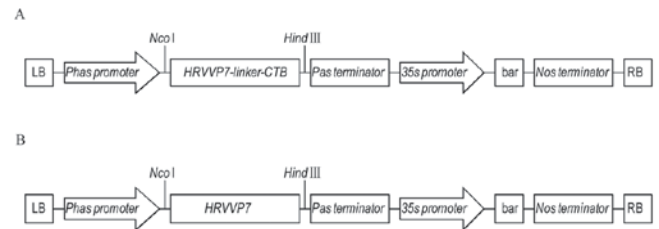

Figure 1. Schematic of plant expression plasmids pPHAP1301-HRVVP7-CTB and pPHAP1301-HRVVP7. *bar* encodes a resistance marker for glufosinate selection. (A) *HRVVP7* coding sequence. (Gly$_4$Ser)$_3$ is the linker between *HRVVP7* and *CTB*. (B) *HRVVP7* coding sequence. HRVVP7, human rotavirus VP7; CTB, cholera toxin B subunit; *Phas,* β-phaseolin storage protein; *35s, Cauliflower mosaic virus;* LB, left border; RB, right border; *nos,* nopaline synthase.

plasmid using the *Agrobacterium tumefaciens* transformation methodology with floral dipping. The presence of the HRVVP7-linker-CTB or HRVVP7 sequence in independent T3 glufosinate-resistant lines was confirmed using RT-PCR with RNA as the template. Six of nine glufosinate-resistant lines yielded bands of the expected 1,428 bp (Fig. 2A) when the HRVVP7-linker-CTB sequence was specifically primed for PCR. These six independent T3 lines (T3-1, T3-2, T3-4, T3-5, T3-6 and T3-8) were used for subsequent analyses. Five of nine glufosinate-resistant lines yielded bands of the expected 1,011 bp (Fig. 2B) when the HRVVP7 sequence was specific primed for PCR. These five independent T3 lines (T3-2, T3-3, T3-5, T3-7 and T3-8) were used for subsequent analyses.

Western blotting was used to examine HRVVP7-linker-CTB and HRVVP7 expression. A 52 or 38 kDa protein was detectable in the transgenic *A. thaliana* seeds with an HRVVP7-specific antibody, indicating that plant-expressed HRVVP7-linker-CTB and HRVVP7 proteins retained the antigenicity of the native rotavirus HRVVP7 protein (Fig. 2C and D). Consistent with this, no bands were present at these positions in the wild type seed samples (Fig. 2C and D).

An ELISA assay was performed to quantify HRVVP7 expression. The results revealed that levels of HRVVP7 in the *A. thaliana* seeds from the HRVVP7-linker-CTB transgenic lines ranged from 3.00 to 52.65 μg/g and accounted for 0.03-0.39% of TSP. Line T3-4 had the highest expression, ~52.65 μg of HRVVP7 protein per gram of seeds. In the HRVVP7 transgenic lines, levels of HRVVP7 in the *A. thaliana* seeds ranged from 9.00 to 43.3 μg/mg and accounted for 0.06-0.31% of TSP. Line T3-8 again had the highest expression, ~43.3 μg of HRVVP7 protein per gram of seeds (data not shown).

Antibody titers in animals in response to plant-expressed pPHAP1301-HRVVP7-linker-CTB and pPHAP1301-HRVVP7. Saliva samples were collected and tested for HRVVP7-specific IgA titers following immunization on days 11, 25, 39 and 53 (Fig. 3A). Anti-HRVVP7 IgA antibodies were detected in mouse saliva collected from the HRVVP7-linker-CTB and HRVVP7 groups from day 11. The anti-HRVVP7 IgA antibody titers increased gradually from day 25 and reached a maximum on day 53. Anti-HRVVP7 IgA antibody titers in the saliva samples from the HRVVP7-linker-CTB group ranged from 1:580 to 1:700 and were significantly higher compared

with the HRVVP7 group (P<0.001; Fig. 3A), which ranged from 1:450 to 1:530. The anti-HRVVP7 IgA antibody titers from feces followed a similar pattern to the saliva (Fig. 3B). On day 53 following the first immunization the anti-HRVVP7 IgA antibody titers in the feces samples ranged from 1:490 to 1:560 in the HRVVP7-linker-CTB group and 1:380 to 1:450 in the HRVVP7 group. Serum anti-HRVVP7 IgG antibody titer in the HRVVP7-linker-CTB group ranged from 1:9,600 to 1:10,300, which was significantly higher compared with the HRVVP7 group (P<0.001; Fig. 3C). Similarly, the anti-HRVVP7 IgA antibody titer in the small intestinal mucosa measured at week 8 ranged from 1:560 to 1:620 in the HRVVP7-linker-CTB group, significantly higher compared with the HRVVP7 group (P<0.001; Fig. 3D). No reactivity with HRVVP7 was observed in the sera, feces saliva and small intestinal mucosa of mice in the negative control group (Fig. 3A-D).

Effect of serum anti-HPVVP7 IgG in neutralizing the virus in vitro. To examine the functional activity of the anti-rotavirus antibodies detected following the oral administration of plant-expressed HRVVP7 or HRVVP7-linker-CTB protein, *in vitro* rotavirus neutralization assays were performed using sera collected on day 39 post-immunization (Fig. 4A-D). The results revealed the sera from mice immunized with HRVVP7-linker-CTB protein more efficiently neutralized the virus than the sera from mice immunized with HRVVP7 protein from transgenic *A. thaliana* seeds.

Observation and statistical analysis of mouse pups following viral challenge. To evaluate the protection afforded by the oral vaccine against rotavirus-induced diarrhea, a viral challenge was performed in passively immunized suckling mice at 5 days old. The number of mouse pups with diarrhea following viral challenge was counted by evaluating the criteria for diarrhea (13). The incidence, severity and duration of diarrheic symptoms were significantly lower in the HRVVP7 and HRPPV7-linker-CTB groups compared with the negative control group from day 1 to day 8 (P<0.001; Fig. 4E). These observations indicated that the mice that received the plant-expressed HRVVP7 protein effectively provided passive protection to their offspring from rotaviral infection. These results suggest that oral delivery of plant-expressed HRVVP7-linker-CTB or HRVVP7 to female mice provided passive protection from rotaviral challenge to their suckling neonatal progeny.

Discussion

In the present study, *A. thaliana* seeds were used as a bioreactor to produce recombinant HRVVP7-linker-CTB or HRVVP7. TSP was extracted from the seeds of transgenic *A. thaliana* plants and the immunogenicity of the transgenic proteins was demonstrated by the production of specific antibodies, the neutralizing ability of which was tested using SA11 infection in cell culture and by passive immune protection afforded the offspring of HRVVP7-immunized mice against SA11 infection.

Rotaviruses are the leading cause of severe gastroenteritis and dehydrating diarrhea in young children and animals worldwide (34). A number of newborn animals are susceptible to
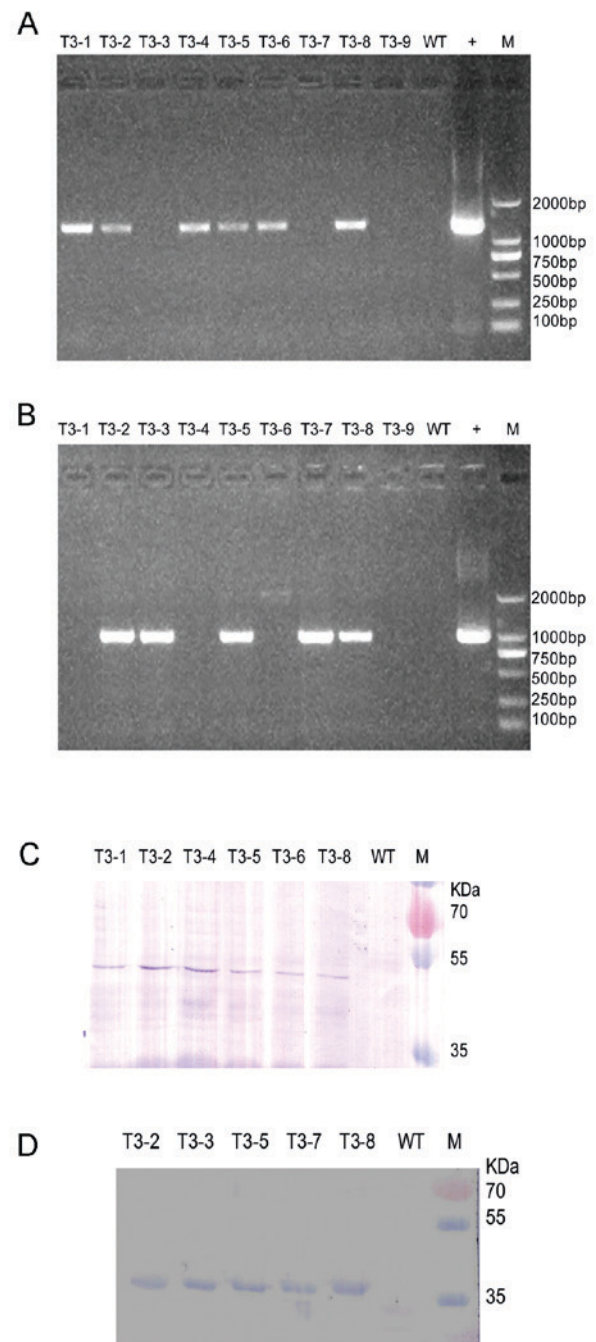

Figure 2. HRVVP7-CTB molecular detection was performed using polymerase chain reaction and western blotting. Agarose gel electrophoresis was used to screen for positive transgenic lines following amplification. (A) HRVVP7-CTB fusion transgenic *Arabidopsis thaliana* lines. pUC19-HRVVP7-linker-CTB plasmid was used as the positive control (+). (B) HRVVP7 transgenic *A. thaliana* lines. pPHAP1301-HRVVP7 plasmid was used as the positive control (+). Proteins expressed by transgenic *A. thaliana* were assessed using western blotting. (C) HRVVP7-linker-CTB and (D) HRVVP7 expressed in seeds of T3 transgenic *A. thaliana* lines. HRVVP7, human rotavirus VP7; CTB, cholera toxin B subunit; WT, wild type *A. thaliana;* +, positive control; M, marker.

rotavirus-induced diarrhea only during the first weeks of life, making it difficult to actively immunize the animals prior to exposure to the pathogen (34). Because female mice are able to passively transfer antibodies to their pups, it is very important
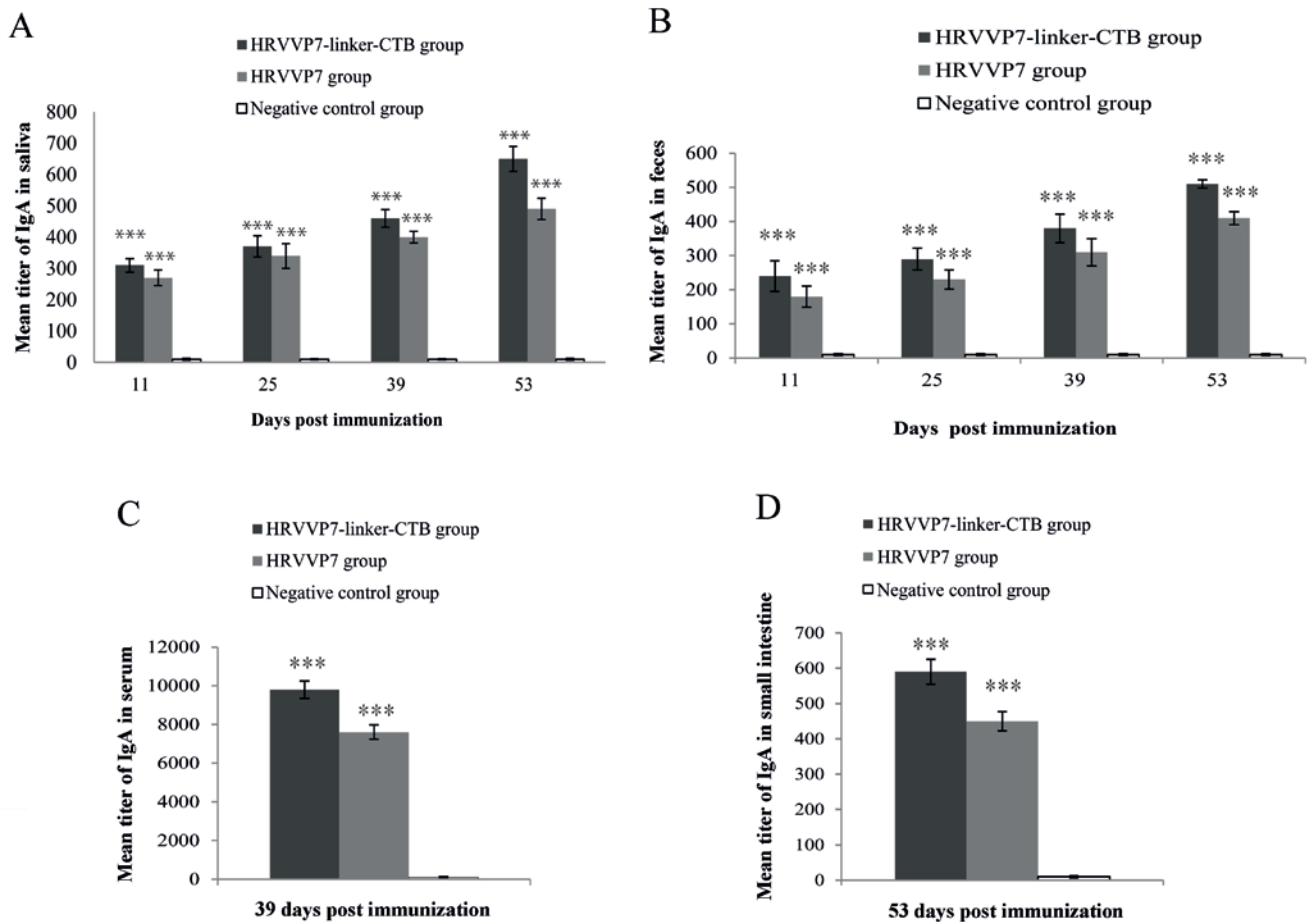
Figure 3. Anti-HRVVP7 antibody titers in mice were measured using ELISA following oral immunization with wild type or transgenic *A. thaliana*. Levels of IgA in (A) saliva and (B) feces were measured at days 11, 25, 39 and 53 following the first immunization. (C) HRVVP7-specific serum IgG at 39 days following the first immunization. (D) Levels of specific IgA in the small intestine at 53 days following the first immunization. Data is presented as the inverse mean antibody titer. ***P<0.001 vs. negative control. HRVVP7, human rotavirus VP7; Ig, immunoglobulin; CTB, cholera toxin B subunit.

to develop a rotavirus vaccine that can induce efficient passive protection against viral infection (35,36).

The expression of heterologous proteins in plants to produce various target antigens and antibodies has previously been explored (15,37-44). Although the prokaryotic expression of VP7 has already been reported (45), recombinant HRVVP7 was successfully expressed in *A. thaliana* for the first time in the present study. A strong tissue-specific promoter (β-phaseolin promoter) was used in the present study to resolve the common problem of low-level expression in transgenic plant bioreactors (46). Using this approach, the production of HRVVP7 reached 0.39% of TSP; however, this yield is still much lower than that reported in *Escherichia coli* (47). Despite the relatively low yield of HRVVP7, the expression strategy used in the present study has commercial potential. Furthermore, the shell of the plant seed is able to protect the exogenous protein from degradation in the gastrointestinal tract, thus rendering plant-expressed vaccines suitable for oral application. A previous study demonstrated that an oral vaccine fused with the mucosal adjuvant CTB induced a mucosal immune response against *Helicobacter pylori* infection in a BALB/c mouse model (48). A similar approach was used in the present study, fusing HRVVP7 to CTB and expressing

the fusion protein in *A. thaliana* plants. In future studies, the fusion protein should be expressed in edible plants, including tomato and carrot, to develop an oral HRVVP7 vaccine.

Interestingly, a previous study indicated that the immune responses of mice fed rotavirus VP7 transgenic plants did not differ significantly from those of mice fed only transgenic VP7 potatoes or transgenic VP7 potatoes plus bacterially expressed recombinant CTB, a kind of mucosal adjuvant (49). The reasons for these similar immune responses may involve the methods used (CTB was dissolved in PBS and placed on potato tuber slices prior to ingestion. so the CTB protein may have been digested in the gastrointestinal tract) or they may have arisen because the transgenic plant itself has a mucosal adjuvant activity associated with its cell wall. An antigen co-expressed with CTB is ideal because it has been reported that the fusion of CTB to pathogenic antigens increases the adjuvanticity of CTB more than 10,000-fold (50). In the present study, the immunological responses of mice following the administration of TSP from transgenic *A. thaliana* seeds expressing HRVVP7-linker-CTB were improved compared with the negative control group. The titers of anti-HRVVP7-linker-CTB IgA in the saliva, small intestines, feces and serum were higher than those specific to HRVVP7 in the immunized mice. To investigate the functional activity
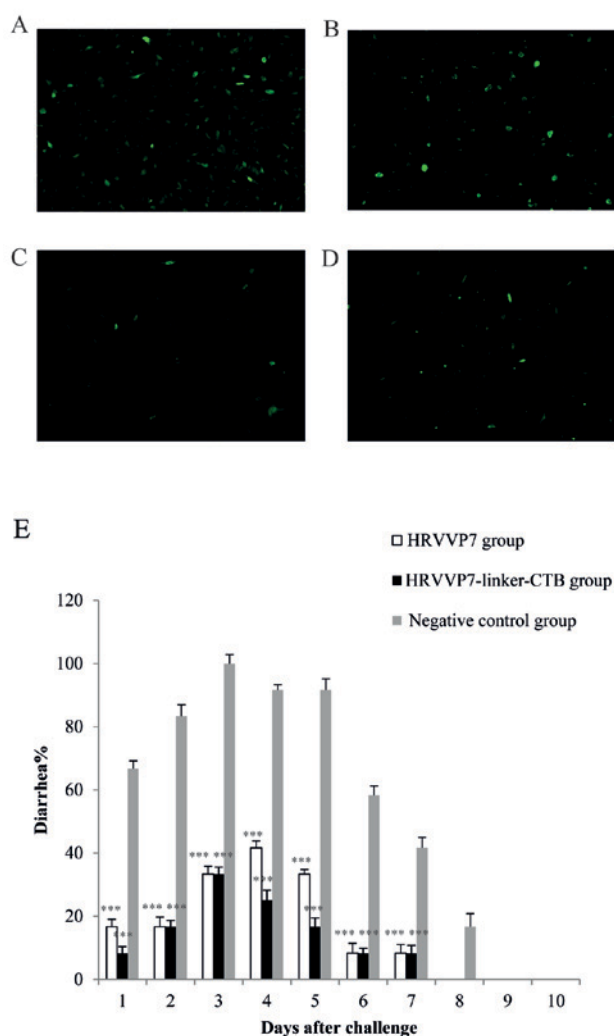
Figure 4. Immunohistochemical analysis and viral challenge. Immunofluorescent analysis of the virus-neutralizing effects of IgG antibodies directed against HRVVP7. (A) Negative control group, (B) HRVVP7-immunized group at 1:42 dilution, (C) HRVVP7-linker-CTB-immunized group at 1:42 dilution and (D) HRVVP7-linker-CTB-immunized group at 1:67 dilution. Magnification, x125. (E) Incidence of diarrhea in neonatal suckling mice following challenge with simian rotavirus SA11. ***P<0.001 vs. negative control. HRVVP7, human rotavirus VP7; Ig, immunoglobulin; CTB, cholera toxin B subunit.

of the anti-rotavirus antibodies detected following the oral administration of plant-expressed HRVVP7-linker-CTB or HRVVP7 protein, rotavirus neutralization assays were performed *in vitro* using serum. The results confirmed that HRVVP7-linker-CTB expressed by transgenic plants induced functionally active anti-rotavirus serum antibodies more efficiently than the similarly expressed HRVVP7. Immunization with HRVVP7-linker-CTB-containing TSP extracts from transgenic *A. thaliana* seeds also passively protected the mouse offspring from severe acute diarrhea after following rotaviral challenge more effectively than immunization with similarly expressed HRVVP7.

One limitation of the present study is that the immunological responses to proteins expressed in *A. thaliana* seeds were not compared with those expressed in *E. coli*. Our group intends to perform such comparative analysis in future studies.

To enhance the versatility and efficacy of the HRVVP7 vaccine, an HRV fusion protein should be made from two or more rotavirus structural proteins, including VP4, VP6 and VP7. This allows for further refinement of this rotavirus vaccine.

In conclusion, the results of the present study demonstrate that *A. thaliana* may be successfully transformed with an HRVVP7-encoding plasmid to express the protein in its seeds. In animal tests, HRVVP7 maintained its immunogenicity and the neutralizing activity of HRVVP7 against rotavirus was primarily attributable to IgA and IgG antibodies. Therefore, plants have the potential to be used as expression systems for the development of rotavirus vaccines. It was also demonstrated that HRVVP7-CTB fusion protein exerts a better mucosal adjuvant effect compared with CTB plus HRVVP7.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL, HL and XiaL conceived and designed the experiments. YL, LG, XiuL, YG and WL performed the experiments. JY, FW and XZ analyzed the data. YL, FW and YG wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments complied with the Animal Research: Reporting In Vivo Experiments Guidelines and were approved by the Ethics Committee of the Jilin Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Parashar UD, Gibson CJ, Bresee JS and Glass RI: Rotavirus and severe childhood diarrhea. Emerg Infect Dis 12: 304-306, 2006.
2. Parashar UD, Bresee JS and Glass RI: The global burden of diarrhoeal disease in children. Bull World Health Organ 81: 236, 2003.
3. Charles MD, Holman RC, Curns AT, Parashar UD, Glass RI and Bresee JS: Hospitalizations associated with rotavirus gastroenteritis in the United States, 1993-2002. Pediatr Infect Dis J 25: 489-493, 2006.
4. Madhi SA, Cunliffe NA, Steele D, Witte D, Kirsten M, Louw C, Ngwira B, Victor JC, Gillard PH, Cheuvart BB, *et al*: Effect of human rotavirus vaccine on severe diarrhea in African infants. N Engl J Med 362: 289-298, 2010.
5. Richardson V, Hernandez-Pichardo J, Quintanar-Solares M, Esparza-Aguilar M, Johnson B, Gomez-Altamirano CM, Parashar U and Patel M: Effect of rotavirus vaccination on death from childhood diarrhea in Mexico. N Engl J Med 362: 299-305, 2010.
6. Greenberg HB, Valdesuso J, van Wyke K, Midthun K, Walsh M, McAuliffe V, Wyatt RG, Kalica AR, Flores J and Hoshino Y: Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J Virol 47: 267-275, 1983.
7. Andrew ME, Boyle DB, Coupar BE, Reddy D, Bellamy AR and Both GW: Vaccinia-rotavirus VP7 recombinants protect mice against rotavirus-induced diarrhoea. Vaccine 10: 185-191, 1992.
8. Dyall-Smith ML, Lazdins I, Tregear GW and Holmes IH: Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc Natl Acad Sci USA 83: 3465-3468, 1986.
9. Offit PA, Boyle DB, Both GW, Hill NL, Svoboda YM, Cunningham SL, Jenkins RJ and McCrae MA: Outer capsid glycoprotein vp7 is recognized by cross-reactive, rotavirus-specific, cytotoxic T lymphocytes. Virology 184: 563-568, 1991.
10. Łucka K, Kowalczyk T, Szemraj J and Sakowicz T: Plants as an alternative source of therapeutic proteins. Postepy Hig Med Dosw (Online) 69: 362-373, 2015 (In Polish).
11. Yusibov V and Streatfield SJ: Plant-produced microbial vaccines: Alexander V. Karasev, editor: Current topics in microbiology and immunology 2009; v. 332. Hum Vaccin 6: pii: 12006, 2010.
12. Arntzen CJ: High-tech herbal medicine: Plant-based vaccines. Nat Biotechnol 15: 221-222, 1997.
13. Dong JL, Liang BG, Jin YS, Zhang WJ and Wang T: Oral immunization with pBsVP6-transgenic alfalfa protects mice against rotavirus infection. Virology 339: 153-163, 2005.
14. Zhou B, Zhang Y, Wang X, Dong J, Wang B, Han C, Yu J and Li D: Oral administration of plant-based rotavirus VP6 induces antigen-specific IgAs, IgGs and passive protection in mice. Vaccine 28: 6021-6027, 2010.
15. Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM and Arntzen CJ: Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 4: 607-609, 1998.
16. Arakawa T, Yu J and Langridge W: Synthesis of a cholera toxin B subunit-rotavirus NSP4 fusion protein in potato. Plant Cell Rep 20: 343-348, 2001.
17. Yu J and Langridge WH: A plant-based multicomponent vaccine protects mice from enteric diseases. Nat Biotechnol 19: 548-552, 2001.
18. Kim TG and Langridge W: Assembly of cholera toxin B subunit full-length rotavirus NSP4 fusion protein oligomers in transgenic potato. Plant Cell Rep 21: 884-890, 2003.
19. Choi NW, Estes MK and Langridge WH: Synthesis of a ricin toxin B subunit-rotavirus VP7 fusion protein in potato. Mol Biotechnol 32: 117-127, 2006.
20. Pêra FF, Mutepfa DL, Khan AM, Els JH, Mbewana S, van Dijk AA, Rybicki EP and Hitzeroth II: Engineering and expression of a human rotavirus candidate vaccine in Nicotiana benthamiana. Virol J 12: 205, 2015.
21. Mason HS, Tacket CO, Richter LJ and Arntzen CJ: Subunit vaccines produced and delivered in transgenic plants as 'edible vaccines'. Res Immunol 149: 71-74, 1998.
22. Yang J, Guan L, Guo Y, Du L, Wang F, Wang Y, Zhen L, Wang Q, Zou D, Chen W, *et al*: Expression of biologically recombinant human acidic fibroblast growth factor in Arabidopsis thaliana seeds via oleosin fusion technology. Gene 566: 89-94, 2015.
23. Mikschofsky H, König P, Keil GM, Hammer M, Schirrmeier H and Broer I: Cholera toxin B (CTB) is functional as an adjuvant for cytoplasmatic proteins if directed to the endoplasmatic reticulum (ER), but not to the cytoplasm of plants. Plant Sci 177: 35-42, 2009.
24. Arêas AP, Oliveira ML, Miyaji EN, Leite LC, Aires KA, Dias WO and Ho PL: Expression and characterization of cholera toxin B-pneumococcal surface adhesin A fusion protein in Escherichia coli: Ability of CTB-PsaA to induce humoral immune response in mice. Biochem Biophys Res Commun 321: 192-196, 2004.
25. Guo L, Yin R, Liu K, Lv X, Li Y, Duan X, Chu Y, Xi T and Xing Y: Immunological features and efficacy of a multi-epitope vaccine CTB-UE against H. pylori in BALB/c mice model. Appl Microbiol Biotechnol 98: 3495-3507, 2014.
26. Huston JS, Tai MS, McCartney J, Keck P and Oppermann H: Antigen recognition and targeted delivery by the single-chain Fv. Cell Biophys 22: 189-224, 1993.
27. Zhang X, Henriques R, Lin SS, Niu QW and Chua NH: Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc 1: 641-646, 2006.
28. Weigel D and Glazebrook J (eds): Arabidopsis. A Laboratory Manual. Cold Spring Harbor Laboratory Press 165, 2002.
29. Bradford MM: A raoid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-bye-binding. Anal Biochem 72: 248-254, 1976.
30. Health Union: Guide for the care and use of laboratory animals. NIH Publication No. 85-23, 1996.
31. Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, MacLeod RA, Masters JR, Nakamura Y, Reid YA, Reddel RR and Freshney RI: Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J. Cancer 127: 1-8, 2010.
32. Mi K, Ou X, Guo L, Ye J, Wu J, Yi S, Niu X, Sun X, Li H and Sun M: Comparative analysis of the immunogenicity of monovalent and multivalent rotavirus immunogens. PLoS One 12: e0172156, 2017.
33. Rojas-Mancilla E, Oyarce A, Verdugo V, Morales-Verdejo C, Echeverria C, Velásquez F, Chnaiderman J, Valiente-Echeverría F and Ramirez-Tagle R: The [Mo(6)Cl14]2-cluster is biologically secure and has anti-rotavirus activity in vitro. Molecules 22: pii: E1108, 2017.
34. O'Ryan M: Rotarix (RIX4414): An oral human rotavirus vaccine. Expert Rev Vaccines 6: 11-19, 2007.
35. Offit P and Clark H: Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. J Virol 54: 58-64, 1985.
36. Pérez Filgueira DM, Mozgovoj M, Wigdorovitz A, Dus Santos MJ, Parreño V, Trono K, Fernandez FM, Carrillo C, Babiuk LA, Morris TJ and Borca MV: Passive protection to bovine rotavirus (BRV) infection induced by a BRV VP8* produced in plants using a TMV-based vector. Arch Virol 149: 2337-2348, 2004.
37. Mason HS, Lam D and Arntzen CJ: Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 89: 11745-11749, 1992.
38. Haq TA, Mason HS, Clements JD and Arntzen CJ: Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268: 714-716, 1995.
39. Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK and Arntzen CJ: Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci USA 93: 5335-5340, 1996.
40. Hiatt A, Caffferkey R and Bowdish K: Production of antibodies in transgenic plants. Nature 342: 76-78, 1989.
41. Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K and Lehner T: Generation and assembly of secretory antibodies in plants. Science 268: 716-719, 1995.
42. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A and Legocki AB: A plant-derived edible vaccine against hepatitis B virus. FASEB J 13: 1796-1799, 1999.
43. Fischer R, Stoger E, Schillberg S, Christou P and Twyman RM: Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 7: 152-158, 2004.
44. Mason HS, Warzecha H, Mor T and Arntzen CJ: Edible plant vaccines: Applications for prophylactic and therapeutic molecular medicine. Trends Mol Med 8: 324-329, 2002.
45. Perez CA, Eichwald C, Burrone O and Mendoza D: Rotavirus vp7 antigen produced by Lactococcus lactis induces neutralizing antibodies in mice. J Appl Microbiol 99: 1158-1164, 2005.

46. Perez CA, Eichwald C, Burrone O and Mendoza D: Rotavirus vp7 antigen produced by Lactococcus lactis induces neutralizing antibodies in mice. J Appl Microbiol 99: 1158-1164, 2005.
47. Yuan LY, Liu Y, Li CH, Sun MS and Dai CB: Expression in Escherichia coli and immunogenicity of rotavirus VP7. Sheng Wu Gong Cheng Xue Bao 17: 145-149, 2001 (In Chinese).
48. Guo L, Liu K, Xu G, Li X, Tu J, Tang F, Xing Y and Xi T: Prophylactic and therapeutic efficacy of the epitope vaccine CTB-UA against Helicobacter pylori infection in a BALB/c mice model. Appl Microbiol Biotechnol 95: 1437-1444, 2012.
49. Wu YZ, Li JT, Mou ZR, Fei L, Ni B, Geng M, Jia ZC, Zhou W, Zou LY and Tang Y: Oral immunization with rotavirus VP7 expressed in transgenic potatoes induced high titers of mucosal neutralizing IgA. Virology 313: 337-342, 2003.
50. George-Chandy A, Eriksson K, Lebens M, Nordström I, Schön E and Holmgren J: Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. Infect Immun 69: 5716-5725, 2001.