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

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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 HRVVVP7-cholera toxin B subunit (CTB) fusion protein was expressed in Arabidopsis thaliana. To determine the adjuvant effect of HRVVVP7-CTB, HRVVVP7 without CTB was expressed in the same manner. HRVVVP7-CTB accounted for 0.39% of the total soluble protein (TSP) in the transgenic seeds and 52.65 µg/g of HRVVVP7 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 HRVVVP7. Antibody titers were highest in mice orally immunized with the plant-expressed HRVVVP7-CTB protein, whereas HRVVVP7-CTB-specific IgG neutralized the rotavirus. Suckling pups born from dams immunized with the HRVVVP7-CTB fusion protein were protected against challenge with virulent rotavirus. The results of the present study suggest that the HRVVVP7-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 parenterally 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
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/487821/), respectively and modified by codon optimization based on the codon usage of plants by Genewiz, Inc. (Suzhou, China). A 15-residue linker coding sequence (GlySer), 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'-CATGCCATGGGATACTGCGCAGAACTCATG-3' and reverse, 5'-CCCAGCTTTAATCTTATAAGTAGAAAGCAGC-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 Biotechnology 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 expression vectors. The T3 seed lines were confirmed by PCR and 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-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.

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 Tris-Cl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 14 mM mercaptoethanol and 1 mM 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 pPHAPI301-HRVVP7-linker-CTB and pPHAPI301-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 CTTAGTTAGCCATAGAGATACGCGG-3’; and forward, 5’-CATGCCATGGGAATCATCCGCAAACTCATG-3’ and reverse, 5’-CCCAAGCTTAAACTCTATAGAAG 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; 1:1,000 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 H₂SO₄ 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 (A₄₉₀) that was at least two-fold higher than the mean A₄₉₀ 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% CO₂. 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-HRVVVp7-CTB or pPHAP1301-HRVVVp7 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-HRVVVp7-linker-CTBandpPHAP1301-HRVVVp7.** Saliva samples were collected and tested for HRVVp7-specific IgA titers following immunization on days 11, 25, 39 and 53 (Fig. 3A). Anti-HRVVVp7 IgA antibodies were detected in mouse saliva collected from the HRVVp7-linker-CTB and HRVVp7 groups from day 11. The anti-HRVVVp7 IgA antibody titers increased gradually from day 25 and reached a maximum on day 53. Anti-HRVVVp7 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 HRVP7-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 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...
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...
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.

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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.
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