SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

January 2011 Vol.54 No.1: 82–89
doi: 10.1007/s11427-010-4104-3

Immunogenicity and virus-like particle formation of rotavirus capsid proteins produced in transgenic plants

YANG YanMei[1,2,3], LI Xia[4], YANG Hui[4], QIAN Yuan[5], ZHANG You[5], FANG RongXiang[1,2*] & CHEN XiaoYing[1,2*]

[1]*State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;*
[2]*National Plant Gene Research Center, Beijing 100101, China;*
[3]*Graduate University of Chinese Academy of Sciences, Beijing 100039, China;*
[4]*Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China;*
[5]*Laboratory of Virology, Capital Institute of Pediatrics, Beijing 100020, China*

Received April 30, 2010; accepted May 9, 2010; published online November 19, 2010

The human pathogen, group A rotavirus, is the most prevalent cause of acute infantile and pediatric gastroenteritis worldwide, especially in developing countries. There is an urgent demand for safer, more effective and cheaper vaccines against rotavirus infection. Plant-derived antigens may provide an exclusive way to produce economical subunit vaccines. Virus-like particles, constituting viral capsid proteins without viral nucleic acids, are considered a far safer candidate compared with live attenuated viral vaccines. In this study, the rotavirus capsid proteins VP2, VP6 and VP7 were co-expressed in transgenic tobacco plants, and their expression levels, formation of rotavirus-like particles (RV VLPs) and immunogenicity were extensively studied. Quantitative real-time RT-PCR and Western blot analysis revealed that the expression level of *vp6* was the highest while *vp7* was expressed at the lowest levels. The RV VLPs were purified from transgenic tobacco plants and analyzed by electron microscopy and Western blot. Results indicated that the plant-derived VP2, VP6 and VP7 proteins self-assembled into 2/6 or 2/6/7 RV VLPs with a diameter of 60–80 nm. When orally delivered into mice with cholera toxin as an adjuvant, the total soluble protein extracted from transgenic tobacco plants induced rotavirus-specific antibodies comparable with those of attenuated rotavirus vaccines, while VP 2/6/7 induced higher serum IgG and fecal IgA titers compared with VP 2/6.

rotavirus, virus-like particles, transgenic plant, oral vaccine, immunogenicity

Citation: Yang Y M, Li X, Yang H, *et al*. Immunogenicity and virus-like particle formation of rotavirus capsid proteins produced in transgenic plants. Sci China Life Sci, 2011, 54: 82–89, doi: 10.1007/s11427-010-4104-3

Rotavirus (RV) is the leading cause of severe infantile gastroenteritis, accounting for more than 125 million cases of diarrhea and causing 600000 deaths per year, mainly in developing countries [1]. In developed countries, dehydration in children suffering diarrhea caused by rotavirus infection is a common cause of hospitalization. The Global Alliance on Vaccines and Immunizations (GAVI) identified a rotavirus vaccine as one of the top three priority vaccines for international development.

Rotavirus belongs to the virus family Reoviridae, and is composed of three concentric protein layers surrounding 11 segments of double-stranded RNA (dsRNA), each coding for products that are either viral structural proteins (VPs) or non-structural proteins (NSPs). 60 dimers of VP2 comprise the inner core. The middle layer is formed by 260 trimers of VP6, which accounts for 50% of the total virus protein mass, is highly immunogenic and contains T-cell antigenic determinants shared by various rotavirus types within group A [2,3].

*Corresponding author (email: fangrx@im.ac.cn; chenxy@im.ac.cn)

The outer layer is composed of 260 trimers of the glycosylated protein, VP7, with spikes formed by the 60 trimers of VP4. Individual VP4 or VP7 is able to induce the neutralizing antibody, which can passively protect mice against rotavirus challenge [4–8], so they are considered as targets for protective immunity in rotavirus vaccine development.

The first RV vaccine, RotaShield, was composed of an attenuated reassortment strain and was licensed in the United States in 1998 [9]. However, the vaccine was withdrawn from the market in less than a year because its delivery was associated with intussusception [10]. Recently, two RV vaccines, RotaTeq and Rotarix, were developed and shown to efficiently protect against severe diarrhea, however, they are produced using live attenuated RV strains [11–15]. Besides the side effects, live attenuated vaccines have the risk of possible virulence reversion. Intense efforts have been made for decades to develop more efficient and safer vaccines against RV, including DNA vaccines, but the IgG induced by DNA vaccination coding for VP4 or VP7 fails to protect mice against RV challenge [16].

A novel vaccine strategy utilizing virus-like particles (VLPs) is developing rapidly. Virus-like particles consist of virus capsid proteins, hence they are morphologically similar to the native virus particles, and can trigger B and T cell responses [17,18]. Their compact structure likely protects them from degradation by digestive enzymes in the gut when orally delivered, as is the case with Norwalk VLPs [19]. Additionally, VLPs contain no genome, meaning that they are safer to use than conventional vaccines.

Rotavirus-like particles (RV VLPs) have the potential to be highly immunogenic and safe vaccine candidates against RV infection, and have received substantial attention in recent years. Self-assembled RV VLPs composed of different combinations have been produced by co-expressing the RV capsid proteins VP2, VP6 and VP7 with or without VP4 in baculovirus-infected insect cells. The 2/6-VLPs in Freund's adjuvant administered parenterally to rabbits afforded protection against RV challenge [20]. When administered intranasally (i.n.) with a mutant *Escherichia coli* heat-labile toxin (LT-R192G) or parenterally with QS-21, the 2/6-VLPs conferred full protection in adult mice against RV infection [21,22]. In a gnotobiotic pig model, which is an RV disease model that most closely mimics the human situation in terms of pathogenicity, i.n. immunization with 2/6-VLPs did not confer protection against diarrhea upon challenge [23,24]. The 2/6/7- and 2/4/6/7-VLPs can prime the immune system against a broad range of homotypic or heterotypic RV challenges and induce anamnestic neutralizing antibody responses in mice and rabbits [25,26]. Additionally, i.n. immunization of mice with 2/6/7-VLPs results in passive protection of pups against RV infection [27]. However, the cost of production and delivery using a baculovirus expression system to produce RV VLPs, is too high for large-scale application, especially in poorer countries and areas.

Expression of foreign antigens in plants provides an efficient way to produce economical subunit vaccines, and plant-derived oral vaccines have been suggested as particularly suitable for use, especially in poorer regions, due to ease of production, handling, and administration, without risk of being contaminated with animal pathogens [28–31]. At present the VLPs of several viruses have been produced in plants successfully, for example, human papillomavirus-like particles and Norwalk VLPs in transgenic tobacco and potato plants, respectively [32–34]; cottontail rabbit papillomavirus-like particles in tobacco plants [35]; hepatitis B virus (HBV) VLPs in tobacco, soybean and potato plants [36,37]; rotavirus 2/6 VLPs in tomato plants [38]; recombinant HIV-1/HBV VLPs in tobacco and *Arabidopsis* plants [39]. In this study, we co-expressed three capsid proteins, VP2, VP6 and VP7 of group A RV in tobacco plants. Plant extract-stimulated RV-specific serum IgG and intestinal IgA in mice when delivered orally with cholera toxin (CT) and a portion of plant-expressed RV capsid proteins could assemble into double- and triple-layered RV VLPs that were morphologically and physically similar to native RV particles.

1 Materials and methods

1.1 Virus RNA extraction and plasmid construction

Human group A RV (P[8]G1) dsRNA was extracted from stool specimens (T114) of infected children in Beijing, China. A 500 μL stool suspension was adjusted to contain 1% sodium dodecyl sulfate and incubated at 56°C for 30 min, centrifuged at 15294×*g* for 5 min at room temperature, and the supernatant transferred to a new tube and extracted once with phenol:chloroform (1∶1). The aqueous phase was precipitated with two volumes of ethanol at –70°C overnight, centrifuged at 15294×*g* for 15 min at 4°C. The RV dsRNA precipitate was resuspended in 100 μL water, and the concentration was determined by absorbance at 260 nm. The cDNAs of *vp2*, *vp6* and *vp7* were synthesized by reverse transcriptase, utilizing RV dsRNA and the primers vp2c, vp6c, vp7c (Table 1) and then cloned into plasmid pTZ18 at the *Sma* I site. Using the resultant plasmids as templates, full-length *vp2*, *vp6* and *vp7* with *Eco*R I and *Hin*d III sites added at both terminals were obtained by polymerase chain reaction (PCR) using primers vp2p, vp6p, vp7p (Table 1) and subcloned at the same sites into the pBI121 binary plasmid, downstream of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Figure 1). The resultant plasmids were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

1.2 Plant transformation and PCR analysis

The leaf discs of axenic tobacco plants (*Nicotiana tabacum*

Table 1 Sequences of primers used in this study

Primers	Forward	Reverse	Position
vp2c	5′-GGCTATTAAA GGCTCAATGGCG-3′	5′-GGTCATATCTCCACAGTGGGGTTG-3′	5′UTR-3′UTR
vp6c	5′-CGCTTTAAAACGAAGTCTTCGAC-3′	5′-GGTCACATCCTCTCACTACATC-3′	5′UTR-3′UTR
vp7c	5′-GGCTTTAAAAGAGAGAATTTC-3′	5′-TCACATCATACAATTCTAATC-3′	5′UTR-3′UTR
vp2p	5′-ATATGGATCCATGGCGTACAGGAAGCGCGGAGC-3′	5′-ATATGAGCTCGGCGTTTACAGTTCGTTCATAA-3′	1-2703
vp6p	5′-TAGGATCCATGGAGGTTTTATATTC-3′	5′-TAGAGCTCTTAGTCTGGTCCTCAC-3′	1-1206
vp7p	5′-TAGGATCCATGTATGGTATTGAATATAC-3′	5′-TTGAGCTCCTATACTCTATAATAAAAC-3′	1-981
vp2s	5′-ACAGGAAGCGCGGAACTAAAC-3′	5′-TGCGACTAGCGACTCACGAAG-3′	1604-2254
vp6s	5′-CTCCACAATCTGAAGCGTTA-3′	5′-TGCGGAAATAGTGCATTAACA-3′	329-929
vp7s	5′-AGGATCAATGGACACTG-3′	5′-ATTAAGCCACCAACTTG-3′	180-796
vp2f	5′-ATGGCGTACAGGAAGCGCGGAA-3′	5′-TTTACAGTTCGTTCATAATGC-3′	1-2703
vp6f	5′-ATGGAGGTTTTATATTCATTG-3′	5′-TTAGTCTGGTCCTCACTTAATC-3′	1-1206
vp7f	5′-ATGTATGGTATTGAATATACCAC-3′	5′-CTATACTCTATAATAAAACGCAG-3′	1-981
vp2re	5′-TCTGGCCGGCATAAATTCACAAG-3′	5′-TGCG ACTAGCGACTCACGAAGAAAC-3′	1167-1348
vp6re	5′-GGTACAACACTTTTAAATTTGGATG-3′	5′-GCTAATTTTCTTAACGCTTCAGATT-3′	202-362
vp7re	5′-ACAATCGGGAGAATCAAATAAGTGGA-3′	5′-TCAGCAACCGTTTCAAATGAGTCTAC-3′	573-710
EF	5′-TGGTGTCCTCAAGCCTGGTATGGTTG-3′	5′-ACGCTTGAGATCCTTAACCGCAACATTCTT-3′	124-283

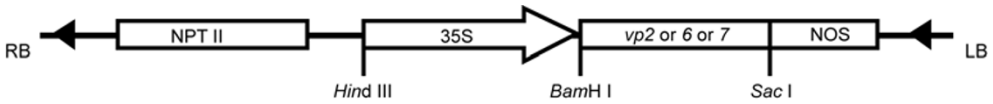

Figure 1 Constructs for plant expression cassettes. The genes *vp2*, *vp6* or *vp7* were cloned into the T-DNA region of the binary vector, pBI121, which contained the neomycin phosphotransferase gene (*nptII*) for kanamycin resistance. 35S, CaMV promoter; NOS, terminator; LB, left border; RB, right border.

cv. SR I) were co-cultured with *A. tumefaciens* strains LBA4404 containing three plant expression vectors simultaneously and grown on Murashige and Skoog (MS) medium containing kanamycin (100 μg mL^{-1}) to regenerate plants. Kanamycin-resistant plants were transplanted to soil and grown under standard conditions in a greenhouse.

Regenerated kanamycin-resistant tobacco plants were further selected by PCR. Genomic DNA of kanamycin-resistant plants was isolated using cetyl trimethyl ammonium bromide (CTAB). The PCRs were performed using the specific primers vp2s for *vp2*, vp6s for *vp6* and vp7s for *vp7* (Table 1). Plasmid pBI221 containing the *vp2*, *vp6* or *vp7* genes and the DNA of non-transgenic tobacco plants were used as the positive and negative controls, respectively. The amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel.

1.3 Reverse transcription PCR (RT-PCR) and quantitative real-time RT-PCR (qPCR)

To detect if genes were correctly transcribed, total plant RNA was extracted from transgenic or non-transgenic tobacco plants using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Exactly 2 μg of total plant RNA was used in a reverse transcription (RT) reaction according to a protocol from Promega (Madison, WI, USA), and 2.5 μL of the RT reaction mixture was used to amplify the full length of each gene by PCR with specific primers vp2f for *vp2*, vp6f for *vp6* and vp7f for *vp7* (Table 1). The amplified products were separated by electrophoresis on a 1% (w/v) agarose gel. For quantitative analysis of the *vp2*, *vp6* and *vp7* RNA transcripts in transgenic tobacco plants, the cDNAs of each transcript were further analyzed by qPCR. A 2 μL volume of the RT reaction mixture was added to a 25 μL PCR mixture containing 12.5 μL Real-time PCR Master Mix (TOYOBO) and 0.3 μmol L^{-1} primers (Table 1). The *Nicotiana benthamiana EF1*α mRNA (GenBank accession No. CN744397) was utilized as an internal control using primers EF (Table 1). The qPCR was carried out on an Opticom II (Bio-Rad, Hercules, CA, USA) using the following thermal cycling profile: 95°C for 1 min, followed by 40 cycles of amplification (95°C for 30 s, 60°C for 30 s and 72°C for 30 s). All samples were run in triplicates.

1.4 Total soluble protein extraction and Western blot analysis

A 1 g mass of leaves from transgenic or non-transgenic tobacco plants was ground in a mortar in the presence of liquid nitrogen. The resulting powder was added to 3.3 mL phosphate-buffered saline (PBS; 0.15% Na_2HPO_4, 0.04% KH_2PO4, 0.61% NaCl, pH 7.2) with 0.1% Triton X-100 and 33 μL protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After gentle vortexing, the mixture was centrifuged at 15294×*g* for 15 min at 4°C and the supernatant was collected. The total soluble protein (TSP) was quantified using

a protein assay kit (Bio-Rad).

For Western blot analysis, 80 μg TSP was separated on a 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore), the membrane was blocked with 3% bovine serum albumin (BSA) for 2 h and sequentially probed with goat anti-RV polyclonal antibody (1∶3000; Abcam) for 1 h. After rinsing the membrane to remove the unbound primary antibody, the membrane was incubated with an alkaline phosphatase-conjugated chicken anti-goat IgG (1:3000; Bios) for 1 h. After rinsing the membrane again, NBT/BCIP (Promega) was added for color development following the directions provided by the company.

1.5 Purification of RV VLPs from transgenic tobacco plants and electron microscopy analysis

To purify RV VLPs, 5 g of leaves from transgenic or non-transgenic tobacco plants was ground to a fine powder in liquid nitrogen and added to 16 mL extraction buffer (10 mmol L^{-1} Tris-HCl, pH 7.5, 140 mmol L^{-1} NaCl, 0.1% Triton X-100) with 160 μL protease inhibitor cocktail (Sigma). The sample was gently mixed by inversion and centrifuged twice at 15294×*g* for 15 min at 4°C, then 4 mL of supernatant was loaded onto an 8 mL 40% sucrose cushion in TNC buffer (10 mmol L^{-1} Tris-HCl, pH 7.5, 140 mmol L^{-1} NaCl, 10 mmol L^{-1} $CaCl_2$) with 80 μL protease inhibitor cocktail (Sigma) and centrifuged in a Beckman SW41Ti rotor at 150000×*g* for 3 h at 4°C. The pellet was resuspended in 500 μL TNC buffer and the supernatant was also collected for Western blot analysis as described above.

For negative staining, resuspended pellets were placed on Formvar-carbon coated copper grids, stained for 10 min with 3% phosphotungstic acid and viewed with a JEM-1400 transmission electron microscope.

1.6 Vaccination

For immunization analysis, 40 RV-negative female BALB/c mice (eight-week-old, purchased from The Academy of Military Medical Sciences, Beijing, China) were divided randomly into five groups and immunized orally with the TSP extracted from transgenic or non-transgenic tobacco plants or PBS in a final volume of 300 μL mixed with 10 μg CT as an adjuvant, or 200 μL live oral attenuated RV vaccine (Lanzhou Institute of Biological Products) on days 0, 7, 14 and 21. Mice were starved overnight and gavaged with 30 μL of 7.5% bicarbonate buffer (Gibco) immediately prior to oral inoculation in order to neutralize stomach acidity. In groups I (VP 2/6) and II (VP 2/6/7) each mouse was administered TSP extracted from VP 2/6 or VP 2/6/7 transgenic tobacco plants, respectively, containing 25 μg VP6. In groups III (WT) and IV (PBS) each mouse was gavaged with TSP extracted from non-transgenic tobacco plants or PBS as a negative control. In group V (RV), each mouse was gavaged with a live oral attenuated RV vaccine. All mice were kept in micro isolator cages (Experimental Animal Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) during the whole experiment, and no clinical change was observed in all mice.

Serum was collected by tail bleeding at 0, 28, 35 and 43 days post-immunization (DPI) and stored at −20°C. Fecal samples collected from individual mice 0, 28, 35 and 42 DPI were made into a 10% (w/v) suspension in 0.01 mol L^{-1} PBS containing a protein inhibitor cocktail (Sigma), and centrifuged to remove fecal solids. Processed fecal samples were stored at −20°C.

1.7 Detection of antibody responses by enzyme-linked immunosorbent assay (ELISA)

Rotavirus-specific total antibodies (IgA, IgM, IgG), IgG or IgA present in mouse serum or fecal samples were measured by ELISA. To measure total antibodies to RV, microtiter plates were coated with 500 ng RV at 4°C overnight, washed three times with PBST (PBS with 0.5% Tween 20), blocked with PBST containing 2% BSA at 37°C for 2 h, and washed three times. Samples were serially diluted two-fold and incubated at 37°C for 2 h. Following another wash, horseradish peroxidase-conjugated goat anti-mouse IgA, IgM, and IgG (ICLLab, 1∶3000 dilution) were added and the plates were incubated at 37°C for 2 h. After a final wash, the substrate OPD was added and incubated for 10 min at 37°C. The reaction was terminated with 2 mol L^{-1} H_2SO_4 and the absorbance at 492 nm (A_{492}) was determined.

For measurement of anti-RV serum IgG and fecal IgA, the coating, blocking, washing, and addition of samples were performed in an identical manner as for the total antibody ELISA described above. After washing away the serum samples, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (ICLLab, 1∶3000) at 37°C for 2 h. After a final wash, OPD was added and the A_{492} determined. Antibody titers were defined as the reciprocal of the highest dilution giving an A_{492} that was at least two-fold the mean A_{492} of samples from the negative controls.

2 Results

2.1 Selection of transgenic plants

In total, 175 independent kanamycin-resistant transgenic lines were obtained. These plants were primarily screened by PCR with specific primers for vp2, vp6 and vp7. We found that the majority of transgenic lines contained two genes, *vp6* and *vp7*, and several plants contained *vp2*, but only one plant contained all three genes. This plant was allowed to self-pollinate to obtain T1 generation plants containing VP 2/6 or VP 2/6/7, which were finally used to

carry out immunization experiments.

2.2 Analysis of RV gene transcripts

Transcription of RV genes *vp2*, *vp6* and *vp7* in transgenic tobacco plants was analyzed by RT-PCR with specific primers for each gene. As Figure 2 shows, full-length *vp2* (2703 bp), *vp6* (1206 bp) and *vp7* (981 bp) were detected in total RNA by RT-PCR, having the same size as the corresponding positive control. By contrast, PCR amplification of total RNA preparations from non-transgenic tobacco plants did not display the relevant bands. The accumulation of *vp2*, *vp6* and *vp7* mRNAs in transgenic tobacco plants was estimated by qPCR. The housekeeping-gene *EF1*α was used as an internal control and the RNA extracted from non-transgenic tobacco plants was used as a negative control. Plasmids containing *vp2*, *vp6* or *vp7* were used as positive controls for each qPCR, respectively. Relative quantity analysis indicated the amount of *vp6* mRNA was 500 times more than that of *vp7* or *vp2*, and the amount of *vp7* mRNA was lowest (Figure 3).

2.3 Analysis of RV proteins produced in tobacco plants

Crude protein extracts from transgenic tobacco leaves were used in the Western blot analysis and probed with anti-RV polyclonal antibody (Figure 4). VP2, VP6 and VP7 were clearly detected in transgenic tobacco plants at their expected sizes of 84 (VP2), 45 (VP6) and 37 kD (VP7), and no relevant band was found in proteins extracted from non-transgenic tobacco plants. Protein quantification was achieved by Western blot techniques. The amount of each protein was normalized to VP7 expressed in *E. coli* at a known protein concentration, which was related to the corresponding band density, allowing a comparison between different protein amounts. The amount of VP6 was greatest, accounting for up to 1.5‰ of the TSP, obviously as a result of its high transcript levels.

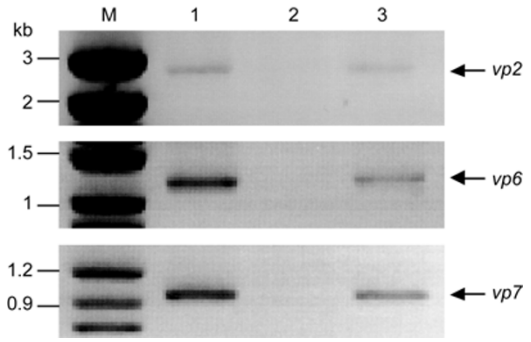

Figure 2 RT-PCR detection of *vp2*, *vp6* and *vp7* mRNAs. Lane 1, PCR products from corresponding plasmid DNAs (positive controls). Lane 2, RT-PCR products from total RNA extracted from non-transgenic tobacco plants (negative controls). Lane 3, RT-PCR products from total RNA of transgenic tobacco plants. M, a molecular weight ladder.

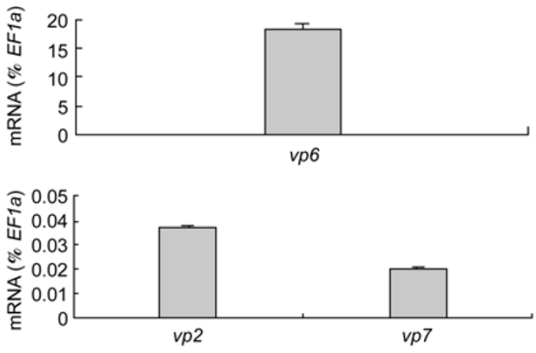

Figure 3 Relative quantification of accumulation of *vp2*, *vp6* and *vp7* mRNAs as measured by qPCR in transgenic tobacco plants. Housekeeping gene *EF1*α was used as an internal control.

2.4 Co-expressed VP2, VP6 and VP7 in transgenic tobacco plants assemble into RV VLPs

To purify RV VLPs, the crude protein extract of transgenic tobacco leaves was centrifuged through a 40% sucrose cushion and the supernatant and pellet were collected. The pellet was resuspended and negatively stained with 3% phosphotungstic acid and observed by electron microscopy. As expected, spherical particles with a 60–80 nm diameter were found (Figure 5A–D), which were very similar in appearance to native RV particles (Figure 5E). No such particle was found in the pellet purified from the protein of non-transgenic tobacco leaves (Figure 5F). This result indicated that co-expressed VP2, VP6 and VP7 proteins in tobacco plant cells could interact with each other and assemble into authentic icosahedral RV VLPs.

The pellet and supernatant obtained above were further analyzed by Western blot with an anti-RV polyclonal antibody. As shown in Figure 6, when the pellet sample was incubated at 37°C for 10 min before loading, no monomer protein was detectable. When the pellet sample was boiled

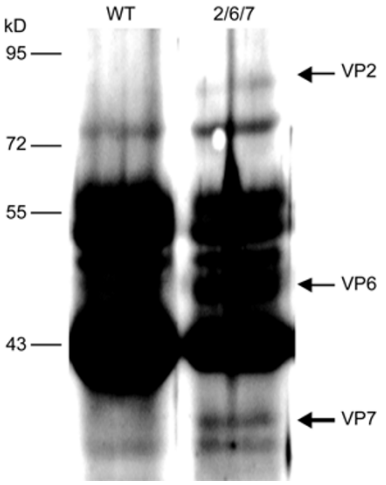

Figure 4 Western blot analysis of TSP in transgenic tobacco plants. TSP was extracted from tobacco plants transformed with *vp2*, *vp6* and *vp7* (2/6/7) or non-transgenic tobacco plants (WT). The protein samples were denatured by heating at 100°C for 10 min before loading and probed with the goat anti-RV polyclonal antibody.

for 10 min before loading, VP2, VP6 and VP7 monomers were reliably detected. The stoichiometric mass ratio of VP2∶VP6∶VP7 in the pellet was 1∶4.26∶0.77, biased from that in the native RV particle, which was 1∶2.8∶2.4 (Table 2). The outer-layer protein, VP7, may only partially exist or be completely absent in some RV VLPs. Obviously, both 2/6 VLPs and 2/6/7 VLPs existed in the pellet, but they could not be distinguished by electron microscopy. Large amounts of VP2, VP6 and VP7 monomers were present in the supernatant (Figure 6), which meant most of the viral capsid proteins did not assemble into RV VLPs. Based on the result of comparing the stoichiometric mass of dissociative RV proteins in the supernatant with that in the pellet, it can be concluded that only a small portion of RV capsid proteins produced in transgenic tobacco plants assembled into RV VLPs.

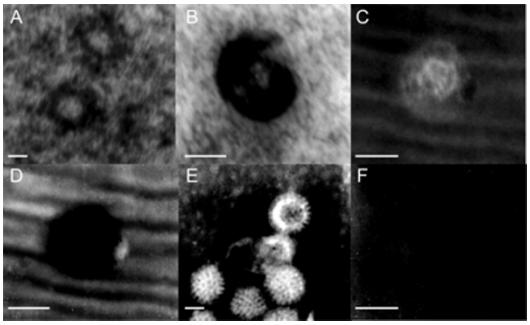

Figure 5 Detection of RV VLP purified from transgenic tobacco plants by electron microscopy. A–D, RV VLPs purified from tobacco plants transformed with *vp2*, *vp6* and *vp7*. E, Native rotavirus particles. F, Negative control from non-transgenic tobacco plants. Scale bar=50 nm.

Table 2 Mass stoichiometric ratio of RV proteins

	VP6∶VP2	VP7∶VP2
In TSP	3.73	1.47
In pellet	4.26	0.77
Native rotavirus particle	2.80	2.40

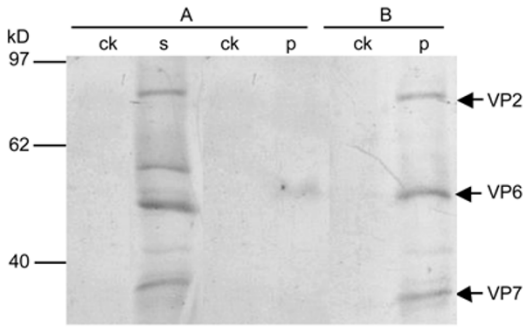

Figure 6 Western blot analysis of RV VLPs purified from transgenic tobacco plants. A, Samples were incubated at 37°C for 20 min before loading. B, Samples were denatured by heating at 100°C for 10 min before loading. s, the sample taken from the supernatant; p, the sample taken from the pellet; ck, control from non-transgenic tobacco plants.

2.5 Rotavirus capsid proteins produced in tobacco plants can induce humoral and mucosal immune responses in orally immunized mice

Rotavirus antibody-negative BALB/c mice orally received four doses of crude soluble leaf extract from either transgenic or non-transgenic tobacco plants mixed with the mucosal adjuvant, CT. Serum and fecal samples were collected on 28, 35 and 43 DPI from experimentally immunized mice and probed for the presence of RV-specific total antibodies by ELISA (Figure 7A and B). At 28 DPI, the total RV-specific antibody titers in serum and feces were low and did not differ significantly among mouse groups, then increased steadily until 43 DPI. At 43 DPI, VP 2/6/7 group mice induced the highest ELISA titer of RV-specific total antibodies in both serum and fecal samples.

Rotavirus-specific serum IgG was evaluated on 43 DPI (Figure 7C). Compared with mice inoculated with the control plant extract, both the VP 2/6 and VP 2/6/7 groups of mice developed significantly high serum IgG titers. Meanwhile, the level of rotavirus-specific IgG antibody detected in the sera of VP 2/6/7 group mice was significantly higher than in the sera of VP 2/6 and RV group mice. The serum IgG titer in the VP 2/6 group was almost as high as that detected in the RV group.

Immunized mice were tested for presence of RV-specific intestinal IgA in processed fecal samples by ELISA (Figure 7D). None of the mice immunized orally with extracts from control tobacco plants developed any RV-specific intestinal IgA responses. However, ELISA titers of mucosal IgA found in RV group mice were significantly higher than that in the VP 2/6 and VP 2/6/7 group mice. We also compared the effect of protein composition of orally administered VPs (VP 2/6 or VP2/6/7) on immunogenicity, and VP 2/6/7 was found to induce higher serum IgG and fecal IgA titers in mice when compared with VP 2/6.

3 Discussion

Vaccination is an efficient form of preventing transmission of infectious diseases. Considerations regarding the suitability of vaccines revolve around their efficiency, safety, cost and delivery. For years, scientists have been seeking ways to produce vaccines with these qualities. Plant-based vaccines may be the solution to these problems, especially in the case of oral vaccines. Compared with prokaryotic expression systems, plants can properly handle the downstream processing of foreign proteins, including phosphorylation, glycosylation and folding, which is important for immunogenicity of an antigen protein. If oral vaccines are produced in edible parts of the plants, they can be delivered by the oral route directly without further processing, such as purification. Plant systems do not harbor human or animal pathogens; therefore, they do not transmit any pathogens

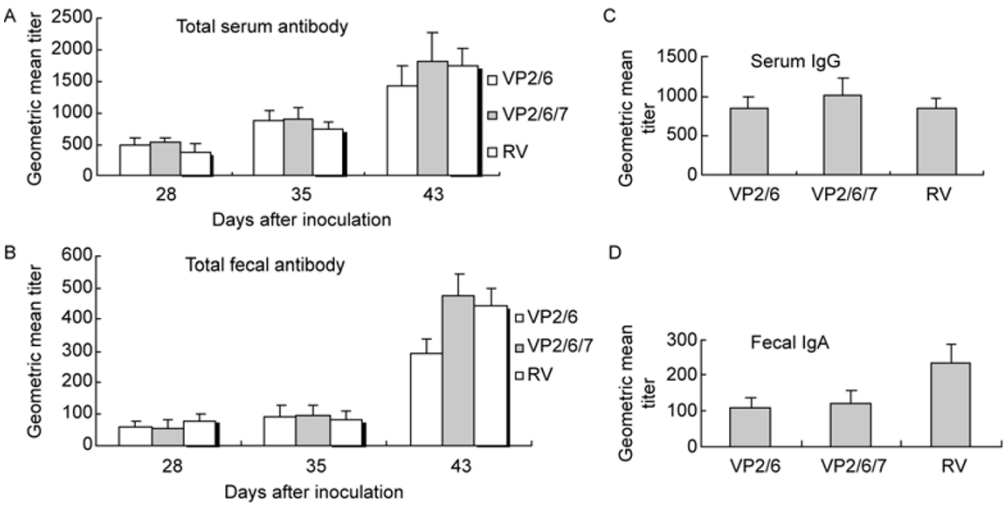

Figure 7 Immune responses in mice following mucosal oral immunization with TSP from transgenic tobacco plants. Total (IgA, IgG and IgM) serum (A) and fecal (B) anti-RV antibodies were measured in each group of mice at 28, 35, 43 DPI; RV-specific serum IgG (C) and fecal IgA (D) were measured in each group of mice at 43 DPI.

that could be damaging to humans.

The advantages of plant based subunit vaccines are incontestable, but many challenges remain, especially the efficiency of the vaccine. Using VLPs as antigens is a new strategy to produce higher efficiency subunit vaccines. VLPs produced in plant cells can provide stronger and longer-lasting vaccines, but the work to produce VLPs in plants is lagging. Up to now, the RV vaccines available on the market are live attenuated vaccines. To produce a safer and cheaper RV vaccine, the plant-based RV VLPs strategy designed by us involved three RV capsid protein genes including *vp2*, *vp6* and *vp7*, which were co-transformed into tobacco plants, and the transgenic plant containing *vp2*, *vp6* and *vp7* was obtained.

Mice orally immunized with TSP extracted from the transgenic tobacco leaves, with CT as an adjuvant, induced RV-specific antibodies comparable to those of an attenuated RV vaccine. The TSP containing VP 2/6/7 induced the highest serum IgG titer, while the replicating attenuated RV vaccine induced the highest intestinal IgA titer. The TSP containing VP 2/6/7 induced higher serum IgG and fecal IgA titers in mice compared with VP 2/6. This result does not exceed our expectations, since the VP7 was absent in the later samples.

The RV VLPs were purified from transgenic tobacco leaves and observed by electron microscopy. Further Western blot analysis indicated the particles comprised 2/6/7- and 2/6-VLPs. VP7-containing RV VLPs have not been obtained in plants previously. Many researchers have successfully produced and purified RV VLPs in insect cells; however, the efficiency of assembly of RV VLPs in insect cells is very low, with only about 15% of the total virus capsid proteins produced in the insect cells participating in the formation of RV VLPs [40,41]. The self-assembly efficiency of 2/6-RV VLPs produced in transgenic plants was even lower [38]. In this study, we came to the same conclusion by comparing the stoichiometric mass of proteins in the supernatant with that in the pellet purified through a sucrose cushion.

One important factor affecting the yield of RV VLPs may be the stoichiometric mass ratio of capsid proteins produced in the plant cell. For efficient RV VLP formation, the ratio of each capsid protein was significantly important [40,41]. In our study, both 2/6 and 2/6/7 RV VLPs were detected in the extract from transgenic tobacco plants. By comparing the stoichiometric mass ratio of VP2:VP6:VP7 produced in tobacco plants compared with that in the native RV particle, we found that VP7 was under-expressed and it could be the limiting protein during the assembly of triple-layered particles. According to studies by Vieira and Carrondo [40,41], the outer layer protein VP7 should be more than VP2 and VP6 thus avoiding accumulation of waste monomers or other protein complexes, such as VP6 trimers [42,43].

In conclusion, we co-expressed the RV capsid proteins VP2, VP6 and VP7 in tobacco plants successfully. The TSP containing VP2/6 or VP2/6/7 was able to induce serum IgG and fecal IgA in mice. VP2, VP6 and VP7 produced in tobacco plants can assemble into VLPs, which are morphologically similar to native RV particles. It was very encouraging that RV VLPs containing VP7, a neutralizing antigen, were found in the transgenic plants, although the amount found was quite low. Since there were not enough RV VLPs available, we could not compare the immunogenicity among the mixture of VP2, VP6 and VP7 proteins, the RV VLPs formed by these three proteins and the attenuated RV. More research is required to improve the formation of the RV VLPs.

This work was supported by the National High Technology Research and Development Program of China (*Grant No. 2007AA100505*).

1 O'Ryan M, Prado V, Pickering L K. A millennium update on pediatric diarrheal illness in the developing world. Semin Pediatr Infect Dis, 2005, 16: 125–136
2 Banos D M, Lopez S, Arias C F, *et al.* Identification of a T-helper cell epitope on the rotavirus VP6 protein. J Virol, 1997, 71: 419–426
3 Estes M K, Crawford S E, Penaranda M E, *et al.* Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. J Virol, 1987, 61: 1488–1494
4 Offit P A, Shaw R D, Greenberg H B. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins vp3 and vp7. J Virol, 1986, 58: 700–703
5 Matsui S M, Offit P A, Vo P T, *et al.* Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. J Clin Microbiol, 1989, 27: 780–782
6 Mackow E R, Vo P T, Broome R, *et al.* Immunization with baculovirus-expressed VP4 protein passively protects against simian and murine rotavirus challenge. J Virol, 1990, 64: 1698–1703
7 Andrew M E, Boyle D B, Coupar B E, *et al.* Vaccinia-rotavirus VP7 recombinants protect mice against rotavirus-induced diarrhoea. Vaccine, 1992, 10: 185–191
8 Both G W, Lockett L J, Janardhana V, *et al.* Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. Virol, 1993, 193: 940–950
9 Kapikian A Z, Hoshino Y, Chanock R M, *et al.* Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children. J Infect Dis, 1996, 174: S65–72
10 Centers for Disease Control and Prevention. Withdrawal of rotavirus vaccine recommendation. Jama, 1999, 282: 2113–2114
11 Madhi S A, Cunliffe N A, Steele D, *et al.* Effect of human rotavirus vaccine on severe diarrhea in African infants. N Engl J Med, 2010, 362: 289–298
12 Richardson V, Hernandez-Pichardo J, Quintanar-Solares M, *et al.* Effect of rotavirus vaccination on death from childhood diarrhea in Mexico. N Engl J Med, 2010, 362: 299–305
13 Vesikari T, Karvonen A, Korhonen T, *et al.* Safety and immunogenicity of RIX4414 live attenuated human rotavirus vaccine in adults, toddlers and previously uninfected infants. Vaccine, 2004, 22: 2836–2842
14 Ruiz-Palacios G M, Perez-Schael I, Velazquez F R, *et al.* Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med, 2006, 354: 11–22
15 Vesikari T, Clark H F, Offit P A, *et al.* Effects of the potency and composition of the multivalent human-bovine (WC3) reassortant rotavirus vaccine on efficacy, safety and immunogenicity in healthy infants. Vaccine, 2006, 24: 4821–4829
16 Choi A H, Basu M, Rae M N, *et al.* Particle-bombardment-mediated DNA vaccination with rotavirus VP4 or VP7 induces high levels of serum rotavirus IgG but fails to protect mice against challenge. Virol, 1998, 250: 230–240
17 Bachmann M F, Rohrer U H, Kundig T M, *et al.* The influence of antigen organization on B cell responsiveness. Science, 1993, 262: 1448–1451
18 Bachmann M F, Zinkernagel R M, Oxenius A. Immune responses in the absence of costimulation: Viruses know the trick. J Immunol, 1998, 161: 5791–5794
19 Jiang X, Wang M, Graham D Y, *et al.* Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol, 1992, 66: 6527–6532
20 Ciarlet M, Crawford S E, Barone C, *et al.* Subunit rotavirus vaccine administered parenterally to rabbits induces active protective immunity. J Virol, 1998, 72: 9233–9246
21 McNeal M M, Rae M N, Conner M E, *et al.* Stimulation of local immunity and protection in mice by intramuscular immunization with triple- or double-layered rotavirus particles and QS-21. Virol, 1998, 243: 158–166
22 O'Neal C M, Clements J D, Estes M K, *et al.* Rotavirus 2/6 viruslike particles administered intranasally with cholera toxin, *Escherichia coli* heat-labile toxin (LT), and LT-R192G induce protection from rotavirus challenge. J Virol, 1998, 72: 3390–3393
23 Yuan L, Geyer A, Hodgins D C, *et al.* Intranasal administration of 2/6-rotavirus-like particles with mutant *Escherichia coli* heat-labile toxin (LT-R192G) induces antibody-secreting cell responses but not protective immunity in gnotobiotic pigs. J Virol, 2000, 74: 8843–8853
24 Yuan L, Saif L J. Induction of mucosal immune responses and protection against enteric viruses: Rotavirus infection of gnotobiotic pigs as a model. Vet Immunol Immunopathol, 2002, 87: 147–160
25 Conner M E, Crawford S E, Barone C, *et al.* Rotavirus subunit vaccines. Arch Virol, 1996, 12: 199–206
26 Jiang B, Estes M K, Barone C, *et al.* Heterotypic protection from rotavirus infection in mice vaccinated with virus-like particles. Vaccine, 1999, 17: 1005–1013
27 Coste A, Sirard J C, Johansen K, *et al.* Nasal immunization of mice with virus-like particles protects offspring against rotavirus diarrhea. J Virol, 2000, 74: 8966–8971
28 Cimolai N. Do mycoplasmas cause human cancer? Can J Microbiol, 2001, 47: 691–697
29 Streatfield S J, Howard J A. Plant production systems for vaccines. Expert Rev Vaccines, 2003, 2: 763–775
30 Tacket C O. Plant-derived vaccines against diarrhoeal diseases. Expert Opin Biol Ther, 2004, 4: 719–728
31 Streatfield S J. Oral hepatitis B vaccine candidates produced and delivered in plant material. Immunol Cell Biol, 2005, 83: 257–262
32 Mason H S, Ball J M, Shi J J, *et al.* Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci USA, 1996, 93: 5335–5340
33 Warzecha H, Mason H S, Lane C, *et al.* Oral immunogenicity of human papillomavirus-like particles expressed in potato. J Virol, 2003, 77: 8702–8711
34 Santi L, Batchelor L, Huang Z, *et al.* An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. Vaccine, 2008, 26: 1846–1854
35 Kohl T, Hitzeroth I I, Stewart D, *et al.* Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: A proof-of-concept study. Clin Vaccine Immunol, 2006, 13: 845–853
36 Mason H S, Lam D M, Arntzen C J. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA, 1992, 89: 11745–11749
37 Huang Z, Elkin G, Maloney B J, *et al.* Virus-like particle expression and assembly in plants: Hepatitis B and Norwalk viruses. Vaccine, 2005, 23: 1851–1858
38 Saldana S, Esquivel Guadarrama F, Olivera Flores T de J, *et al.* Production of rotavirus-like particles in tomato (*Lycopersicon esculentum* L.) fruit by expression of capsid proteins VP2 and VP6 and immunological studies. Viral immunol, 2006, 19: 42–53
39 Greco R, Michel M, Guetard D, *et al.* Production of recombinant HIV-1/HBV virus-like particles in Nicotiana tabacum and *Arabidopsis thaliana* plants for a bivalent plant-based vaccine. Vaccine, 2007, 25: 8228–8240
40 Vieira H L, Estevao C, Roldao A, *et al.* Triple layered rotavirus VLP production: Kinetics of vector replication, mRNA stability and recombinant protein production. J Biotechnol, 2005, 120: 72–82
41 Roldao A, Vieira H L A, Alves P M, *et al.* Intracellular dynamics in rotavirus-like particles production: Evaluation of multigene and monocistronic infection strategies. Proc Biochem, 2006, 41: 2188–2199
42 Petitpas I, Lepault J, Vachette P, *et al.* Crystallization and preliminary X-ray analysis of rotavirus protein VP6. J Virol, 1998, 72: 7615–7619
43 Lepault J, Petitpas I, Erk I, *et al.* Structural polymorphism of the major capsid protein of rotavirus. EMBO J, 2001, 20: 1498–1507