Expression of hemagglutinin protein of rinderpest virus in transgenic tobacco and immunogenicity of plant-derived protein in a mouse model

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Abstract

The use of transgenic plants as a production system for recombinant subunit vaccines has been considered safe and economical compared to cell culture methods. We have exploited this approach to produce rinderpest virus hemagglutinin (H) protein in transgenic tobacco as a model plant for testing the immunogenicity of plant-derived hemagglutinin protein. The transgenic nature of the plants was confirmed by molecular analysis such as gene specific PCR and Southern hybridization using full-length H gene as a probe. The Mendelian pattern of inheritance of the transgene has been demonstrated in T1 generation. The transgenic plants express the H protein of molecular weight 72 kDa. The plant derived H protein is antigenically authentic as revealed by reactivity with H-specific antibodies as well as convalescent sera. The induction of immune response was tested in mice after intraperitoneal immunization with plant-derived H. High titers of antibodies were induced which were H-specific and they neutralized the infectivity of rinderpest virus.

Keywords: Rinderpest virus; Hemagglutinin protein; Transgenic plants; Tobacco transformation

Introduction

Transgenic plants offer an economic alternative to produce recombinant vaccine antigens (Mason and Arntzen, 1995). If the vaccine antigens are produced in the edible parts of the plants, they can be directly consumed, providing an easy way for mass immunization. Being produced in plant system these antigens are safe, as there is no chance of contamination of other pathogens. Storage of plant material is easier compared to cell culture or fermentation-based products, as plant-derived antigens do not need cold chain maintenance. To date, several bacterial and viral antigens of human pathogens (Haq et al., 1995; Huang et al., 2001; Mason et al., 1996; McGarvey et al., 1995; Sandhu et al., 2000; Thanavala et al., 1995) have been expressed in plant systems. Although the vaccine trials in humans are limited (Tacket et al., 1998, 2000), they have demonstrated the induction of oral immune response, thus showing the feasibility of developing plant-based edible oral vaccine. This approach has also been utilized to develop transgenic plants expressing vaccine antigens for animal diseases such as foot and mouth disease and transmissible gastroenteritis (Tuboly et al., 2000; Wigdorovitz et al., 1999a, 1999b).

Rinderpest is an acute, usually fatal, highly contagious viral disease of cloven-hoofed animals, particularly cattle and buffaloes. Although a live attenuated tissue culture-adapted rinderpest vaccine exists (Plowright, 1962), it is not robust. International eradication campaigns are thus successful only in limiting the distribution of the disease. It is still prevalent in many parts of Africa, the Middle East, and South Asia (Barrett and Rossiter, 1999). The host cell membrane-derived envelope of Rinderpest virus (RPV) contains two virus-specific glycoproteins—hemagglutinin (H) and fusion (F) protein. These glycoproteins are highly immunogenic and confer complete protection. Recombinant subunit vaccines, vaccinia/capripox recombinant (Romero et al., 1993, 1994; Yilma et al., 1988) or baculo recombinants, have been produced (Bassiri et al., 1993; Naik et al., 1997). Long-term immunity of recombinant vaccinia virus expressing rinderpest H has been tested in cattle (Ohishi et al., 2000).
Toward the aim of developing edible vaccine for rinderpest, tobacco was chosen as the model plant, since transformation procedures are well standardized and the plant is fast growing. The hemagglutinin gene of rinderpest virus was introduced into tobacco by *Agrobacterium*-mediated transformation or by direct particle bombardment. The integration of H gene into plant genomic DNA was established and the expression as well as its antigenicity was demonstrated. Finally, the immunogenicity of the plant-expressed H protein was tested in a mouse model.

**Results**

**Construction of binary vector**

The recombinant binary vector pBI H was generated by subcloning of full-length (1.9 kb) hemagglutinin gene from RBH 3.4 into pBI121 in place of *uid* gene. Thus, the hemagglutinin gene is under the control of constitutively expressed CaMV 35S promoter and is terminated by NOS terminator sequences (Fig. 1). The T-DNA region contains the *nptII* gene in addition to the hemagglutinin gene, which helps in selection of transformants on medium containing kanamycin.

**Genetic transformation of plants**

Genetic transformation of tobacco with pBI H was achieved by *A. tumefaciens* (EHA 105) or directly by particle bombardment as described under Materials and methods. The putative transformants selected on kanamycin were tested for the presence of transgene by PCR. An amplified product (700 bp) was observed when PCR was performed using *nptII*-specific primers and genomic DNA from transformed plants as template and this product was absent when genomic DNA from nontransformed tobacco plant was used (data not shown). Similarly in *H*-specific PCR, the expected size (1.9 kb) product was obtained with transformed plant DNA but was absent with nontransformed plant (Fig. 2). Twenty-five independent lines generated by *Agrobacterium*-mediated transformation and 6 independent lines produced by particle bombardment showed the presence of transgene. Further, the integration of *H* in the genomic DNA of transformants was analyzed by Southern hybridization. Genomic DNA when digested with *EcoRI* showed the presence of a single band of size >4.6 kb when hybridized with the full-length *H* probe as expected. Since the *EcoRI* site is present only once in T-DNA at the

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**Fig. 1.** T-DNA region of the recombinant binary vector pBI H.

**Fig. 2.** Agarose gel electrophoresis showing amplification of *H* by PCR. Lane M: lambda DNA/ *HindIII* digest; lane 1: genomic DNA from nontransformed tobacco; lanes 2 and 3: genomic DNA from TRB1/2 and TRB1/4; lanes 4–8: TRA1/6, TRA1/9, TRA1/10, TRA2/3, and TRA2/9, respectively.

**Fig. 3.** Inheritance pattern of *nptII* gene in the second generation. (A) Plants transformed by particle bombardment were used for the analysis of *nptII* gene by PCR amplification. (B) Statistical analysis of results was performed using chi-square test.
extreme end (Fig. 1), a fragment carrying H gene will always be equal to or more than 4.6 kb, which is the size of the T-DNA, integrated into the plant genome. *Hin*dIII-digested genomic DNA also showed the presence of a single band, thus demonstrating single-copy insertion in most of the lines (Fig. 3). Signals were absent in genomic DNA digests of nontransformed tobacco plants. These positive lines were allowed to self-pollinate and the seeds were collected. The seeds from lines TRB1/2 and TRB1/4 were germinated in Soilrite. Genomic DNA from approximately 35–40 T1 plants of each line was isolated from the leaves and tested for the presence of transgene by nptII-specific PCR. The ratio of the positive and negative plants with respect to the transgene was subjected to statistical analysis. The results (Fig. 4) clearly indicated a Mendelian segregation pattern.

**Rinderpest virus hemagglutinin protein expression in transgenic plants**

Twenty-five independent lines were tested for the expression of the transgene by Western immunoblotting. The expression of hemagglutinin protein was observed in these lines when monospecific polyclonal antibody to H was used. The percentage of H in total soluble protein was estimated by a double antibody sandwich ELISA employing recombinant secretory H and a monoclonal antibody to H (data not shown) and maximum expression of H up to 0.75% was observed in one of the lines. One line (TRA1/10) expressing the highest amount was used to authenticate the nature of plant-derived hemagglutinin. Immunoblotting using different H-specific antibodies confirmed the authenticity of plant-derived 72-kDa hemagglutinin protein (Figs. 5A–D). PCR-positive T1 plants were also analyzed for the expression of hemagglutinin protein. Protein extracts were made from the leaves and immunoblotted after electrophoretic separation using cross-reactive antibody to H (rabbit polyclonal anti-HN antibody), shown in Fig. 5E. Further, the antigenicity of the plant-derived hemagglutinin (in both T0 and T1 transgenic plants) protein was demonstrated by its reactivity with serum from cattle recovered after rinderpest.
infection (Figs. 6A–C) and with serum from cattle immunized with recombinant H (Figs. 6E and F). Cross-reactivity of plant-derived hemagglutinin was tested by its reactivity to antibody against the HN protein of the closely related Peste des petits ruminants virus (PPRV) and serum from convalescent sheep after infection by PPRV, as shown in Figs. 6G and H, respectively.

Immunogenicity in mice

The immunogenicity of the plant-derived H was studied in a mouse model. Adult female BALB/c mice were immunized intraperitoneally with protein extracts from the transformed (Group 1) and nontransformed tobacco (Group 2) plants. The specificity of the serum collected from all five mice of both groups, over a period of 70 days at weekly intervals was analyzed in ELISA using recombinant secretory H protein (Naik and Shaila, 1997) as antigen. H-specific antibodies were remarkably higher in group 1 mice immunized with transgenic tobacco plant extract than in group 2 mice immunized with nontransformed tobacco plant extract (Fig. 7). The specificity of these antibodies was further confirmed by Western immunoblotting of protein from virus-infected (RPV/PPRV Nig 75/1) or uninfected Vero cell lysates as antigens. A 70-kDa protein corresponding to H was detected only in infected cell lysates and no signals were obtained in uninfected cell lysate when pooled serum from mice immunized with transgenic tobacco expressing H was used as primary antibody (inset, Fig. 7),

Fig. 6. Antigenic authenticity of plant-derived H. (A–C) Reactivity of T₀ and T₁ tobacco-derived H and nontransformed tobacco to convalescent sera obtained from three different buffaloes (Nos. 6, 63, 64), respectively. (D) Reactivity of T₀ and T₁ tobacco-derived H and nontransformed tobacco to normal cattle sera. (E and F) Reactivity to cattle sera immunized with recombinant H. (G and H) Cross-reactivity of T₀ and T₁-derived H and nontransformed tobacco, respectively, with convalescent sera from sheep recovered from PPRV infection and with antibody to PPRV-HN. Circle, triangle, and square represent T₀-derived H, T₁-derived H and nontransformed tobacco, respectively.

Fig. 7. Specific antibody levels in mice after ip immunization with transgenic tobacco leaf extract. The antibody titer is defined as the log of the reciprocal of the highest dilution of the serum which gave optical density twice that given by serum from mice immunized with nontransformed tobacco leaf extract. Inset: immunoreactivity of mice serum with antigens from RPV-infected cell lysate (lane 1), PPRV-infected cell lysate (lane 2), and uninfected cell lysate (lane 3).
thus, demonstrating that the plant-derived H is eliciting a specific antibody response.

The functional activity of these serum was demonstrated by hemagglutination inhibition assay. PPRV has been shown to possess hemagglutination activity (Ramachandran et al., 1995). Serum from mice immunized intraperitoneally with plant-derived H showed inhibition of hemagglutination activity of PPRV, while this inhibition was not observed when serum from mice immunized with nontransformed tobacco plant extract was used (Fig. 7).

The virus neutralization ability of different serum samples was tested in vitro. A significant level of neutralizing and cross-neutralizing antibody titers was detected (Table 1) in the pooled serum derived from all five animals intraperitoneally immunized with transformed tobacco plant extract over a period of 10 weeks postimmunization. However, no neutralization activity against RPV or PPRV was detected in serum from mice following intraperitoneal immunization with nontransformed tobacco plant extracts, indicating protection against infection in vitro by immune serum. These results demonstrate that plant-derived H protein is able to induce functionally active anti-H antibodies in the mouse model upon intraperitoneal immunization.

**Discussion**

As a first step toward generation of a hemagglutinin-expressing transgenic fodder crop, which can be used as a candidate edible vaccine for rinderpest, we developed transgenic tobacco plants expressing rinderpest virus hemagglutinin protein. The antigenic nature of the plant-derived hemagglutinin protein based on its reactivity to H-specific antibodies has been demonstrated. Further characterization of the antibody response to plant-derived hemagglutinin in a mouse model after intraperitoneal immunization shows the induction of neutralizing antibodies in the experimental model system.

**Table 1**

<table>
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<tr>
<th>Weeks postimmunization</th>
<th>RPV</th>
<th>PPRV</th>
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<tr>
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<td>9</td>
<td>320</td>
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*Note.* Virus neutralization and cross-neutralization titers are defined as the reciprocal of the highest dilution of the serum which inhibited cytopathic effect by 50%.

**Table 2**

Hemagglutination inhibition titers of pooled serum from five mice after immunization with transgenic tobacco leaf extract (Group 1) and with nontransformed tobacco leaf extract (Group 2) until 9 weeks postimmunization

<table>
<thead>
<tr>
<th>Weeks postimmunization</th>
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<td>9</td>
<td>640</td>
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*Note.* Serum from Group 2 did not show any inhibition of hemagglutination. Hemagglutination inhibition titer is defined as the reciprocal of the dilution, which showed 50% hemagglutination inhibition.

The stability, solubility, folding, and biological activity of a protein can be affected by glycosylation. Therefore exposure of antigenic determinants of a glycoprotein depend on its glycosylation status. N-linked glycan processing differs in plants and mammals (Oxley and Bacic, 1995). Factors, such as polypeptide folding, could influence the availability of potential N-glycosylation sites in the ER. As a consequence, when multiple N-glycosylation sites are present in a protein sequence, some sites might be glycosylated inefficiently, giving rise to various glycoforms, whereas some others are not used at all (Crofts and De necke, 1998). Thus, the in vivo applications of pharmaceutical recombinant glycoproteins produced in transgenic plants strongly depend on their N-glycosylation patterns, and N-glycosylation of a recombinant therapeutic glycoprotein has to be determined in detail (Bardor et al., 1999). RPV H protein has five potential N-glycosylation sites of which four are glycosylated in infected cells and in mature virions. We have shown the glycosylation of plant-derived hemagglutinin protein by concanavalin A blotting. Although the pattern of glycosylation and its extent has not been determined, it is able to mount significant immune response (Fig. 7), and more importantly, these antibodies are able to neutralize virus infectivity in vitro (Table 1).

Particular antigens are known to induce better immune response in comparison to soluble antigens. Soluble antigens need a very good delivery system that can carry the antigen intact through the full repertoire of degrading conditions of gastrointestinal tract such as acidic pH, proteases, and bile acids. Lipid-based delivery systems, which include ISCOMs, liposomes, and oil-based emulsions, are common. Expression of antigens in plant cells provide bioencapsulation of the antigen, thus enabling the antigen to maintain its potency to induce immune response effectively (Kong et al., 2001). There are few reports describing the immunogenic nature of soluble proteins when expressed in plants and delivered orally (Huang et al., 2001; Wigdorovitz et al.,...
1999a). In the report by Huang et al. (2001) induction of neutralizing antibodies in mice upon intraperitoneal immunization or oral immunization with measles virus hemagglutinin protein expressed in tobacco was demonstrated. VP1 protein of foot and mouth disease virus expressed in plants has been shown to induce protective immune response in mice. This group has used different plants, arabidopsis (Carrillo et al., 1998), alfalfa (Wigdorovitz et al., 1999a), and potato (Carrillo et al., 2001), to express the full-length VP1 and reported induction of specific immune response through intraperitoneal or oral inoculation. More recently, Dus Santos et al. (2002) expressed a small immunogenic region, 135 to 165 aa, of VP1 in alfalfa plants and showed the induction of significant immune response. In contrast to these reports, the spike protein of swine-transmissible gastroenteritis virus (TGEV) expressed in potato plants induced specific immune response when administered either by ip or by oral route but failed to generate neutralizing antibodies (Gomez et al., 2000). Earlier, Gomez et al. (1998) reported that S protein expressed in arabidopsis is able to induce significant levels of neutralizing antibodies when inoculated in mice intraperitoneally. The authors have attributed this difference in induction of neutralizing antibodies to the differences in posttranslational modification of the glycoprotein in different plant species or to the presence of other plant proteins. Different formulations were used to immunize the mice, for arabidopsis-derived protein and potato tuber extract, although the expression level in both plants is reported to be the same (0.02–0.07%). Tuboly et al. (2000) have shown the immunogenicity of S protein expressed in tobacco plants in pigs. The protective ability of tobacco-derived S protein was shown by virus neutralization test.

The antigenicity and immunogenicity of each antigen when expressed in plants need to be tested, as the processing of individual antigen may have different effects. Tobacco is the most preferred model plant to express any foreign protein because of its easy transformation and fast regeneration (Richter and Kipp, 1999). Similar to antigens such as LT-B (Haq et al., 1995), hepatitis B surface antigen (Thanavala et al., 1995), and measles virus hemagglutinin protein (Huang et al., 2001), in the present study the rinderpest virus hemagglutinin gene when expressed in tobacco plants maintained immunogenicity in a mouse model. The results also demonstrate the generation of neutralizing antibodies in mice upon intraperitoneal immunization with crude extract from transgenic tobacco expressing RPV H protein, thus indicating the potential of plant-derived hemagglutinin protein as a vaccine candidate.

Materials and methods

Construction of expression vector (pBI H)

Hemagglutinin gene of rinderpest virus was subcloned in binary vector pBI121. The uid gene of pBI121 is replaced by H gene such that the gene will be under the control of CaMV 35S promoter. pBI121 was digested with Smal and SacI to release the uid gene fragment. Full-length H gene was obtained by EcoRI and BamHI digestion of RBH3.4 plasmid DNA, which contains the full-length DNA for H derived from a cDNA library prepared using total mRNA made in Vero cells infected with the vaccine strain of RPV (RBOK) (kind gift from Dr. T. Barrett, Institute for Animal Health, Pirbright, UK). Both the vector and the insert DNA were end-polished using T4 DNA polymerase and ligated. E. coli DH5α was used for transformation of the ligated DNA. Transformants were selected on kanamycin-supplemented (50 mg/L) medium. Recombinants were checked for the presence of H gene in correct orientation by Xhol digestion, which should give a product of approximately 1.9 kb.

Mobilization of pBI H into Agrobacterium tumefaciens (strain EHA 105)

The freeze–thaw method (Holsters et al., 1978) was used for mobilizing pBI H into A. tumefaciens (EHA 105). Transformants were selected on medium supplemented with kanamycin (50 mg/L) and rifampicin (25 mg/L).

Genetic transformation of tobacco

Transformation of tobacco (Nicotiana tabacum var. Hwana) was performed using the leaf disc method (Horsch et al., 1986). In brief, small pieces of leaves were infected with overnight-grown recombinant Agrobacterium harboring pBI H for 5–10 min, blotted dry, and transferred to regeneration medium (MS1) containing MS (Murashige and Skoog, 1962) salts and vitamins, 3% (w/v) sucrose, 2 mg/L benzy1 aminopurine (BAP), 0.5 mg/L naphthalene acetic acid (NAA), and 0.8% agar. After 2 days of cocultivation leaf discs were washed either with MS liquid or sterile distilled water and transferred to selection medium containing appropriate antibiotics to select putative transformants. Selection medium (MS2) contained MS1 supplemented with 500 mg/L cefotaxime, 100 mg/L kanamycin. Root induction medium (MS3) contained MS salts and vitamins, 3% (w/v) sucrose, 0.1 % indole acetic acid (IAA), 500 mg/L cefotaxime, 100 mg/L kanamycin, and 0.8% phytagel. Rooted T0 tobacco plantlets after hardening of roots in liquid MS medium were transferred to Soilrite in small cups and were maintained in the controlled environment of the growth chamber for another 10 days before transfer to the green house. They were allowed to grow to maturity and self-fertilize. Seeds were collected and germinated in the soil and were tested for the presence of transgene in T1 progeny.

Transformation of tobacco with H gene by particle bombardment

Tobacco leaf discs were precultured on regeneration medium (MS1) for 2 days. Microprojectiles were prepared
by coating recombinant pBI H plasmid DNA on 16-µm gold microbeads and the explants were bombarded using the BioRad particle bombardment system as per manufacturer’s instructions. After bombardment, explants were kept in the dark for 2 days and then transferred to selection medium (MS2).

**Polymerase chain reaction (PCR) and Southern hybridization**

The presence of transgene was confirmed by PCR amplification of the nptII and H genes using specific primers. Plant genomic DNA from putative transformants selected on kanamycin-supplemented medium and nontransformed tobacco plants for PCR analysis was isolated by the CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson, 1980). The nptII primer sequences (5'-3') GAG GCT ATT CGG CTA TGA CTG and ATC GGG AGG GGC GAT ACC GTA will amplify an internal fragment of 700 bp. The PCR was performed in a thermal cycler using the following conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, synthesis at 72°C for 1 min and final extension for 7 min at 72°C. The H primer sequences (H1: 5’-GGACTACAGTACCCCGACGT-3' and H2: 3’-TGGTTAGTACTCCATACGCCGGTA-5’) will amplify the full-length (1.9 kb) H gene. The conditions used for the PCR are as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 44°C for 1 min, synthesis at 72°C for 2 min, and final extension for 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

For Southern hybridization 10 µg of total genomic DNA from transgenic plants and nontransformed control plants was digested with EcoRI/HindIII. After agarose gel electrophoresis, DNA was transferred to a nylon membrane (Hybond N+, Amersham, UK) by capillary action overnight (Sambrook et al., 1989). The 1.9-kb fragment of the H gene was obtained by XbaI digestion of pBI H and radio-labeled with [α-32P]dCTP from Amersham (sp act 3000 Ci/mmol) using a Random Priming labeling kit purchased from Amersham as per manufacturer’s instructions. The radiolabeled probe was denatured and used for hybridization after 2 h of prehybridization at 65°C in Church buffer (Church and Gilbert, 1984). Following hybridization at 65°C for 16 h, the membrane was washed for 30 min each in 2× SSC + 0.1% SDS and 1× SSC + 0.1% SDS at room temperature; 0.5× SSC + 0.1% SDS and 0.1× SSC + 0.1% SDS at 65°C. The blot was exposed to phosphor imaging.

**Western blotting**

Leaf tissues (0.1 g) from transformed and nontransformed tobacco plants were ground to a fine powder in liquid nitrogen and resuspended in 500 µL sample buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.2% bromphenol blue). The samples were boiled for 10 min, and the supernatants collected by centrifugation at 13,000 rpm for 15 min. The proteins were subjected to electrophoresis on a 10% SDS–polyacrylamide gel and transferred to a nylon membrane. Bovine gelatin in PBS (3% w/v) containing 0.1% Tween 20 was used for blocking the membrane. The membrane was washed three times with PBS containing 0.1% Tween 20 (PBST). Rabbit polyclonal monospecific anti-H antibody, rabbit polyclonal anti-HN antibody (Devireddy et al., 1998), and mouse monoclonal anti-H antibody (D2E4) (Renukaradhya et al., 2002) were used as primary antibodies in individual blots and the presence of specific antibodies to H by ELISA using

**ELISA**

Total protein from leaves of transformed and nontransformed tobacco plants was isolated using the method of McGarvey et al. (1995). Twofold serial dilution of plant extracts was performed in PBS and coated onto ELISA plates at 4°C overnight. All further incubations were at 37°C for 1 h and the plate was washed three time with PBST. Blocking was done in 0.2% fish gelatin in PBST (v/v). Appropriate antibody dilutions were made for all primary antibodies followed by peroxidase-conjugated anti-cow, anti-mouse antibodies as secondary antibodies. The plates were developed with o-phenylenediamine (OPD) tablets purchased from Sigma for 10–15 min and read at 490 nm.

The amount of H in different transgenic lines was determined by using recombinant H protein in a secretory form (SecH) (Naik and Shaila, 1997), to prepare a standard curve. The level of H in SecH preparation was estimated by separating it on 10% SDS–polyacrylamide gels. The 68-kDa band corresponding to H was analyzed by densitometric scanning. Varying concentrations of H was used in ELISA to make the standard curve (data not shown).

**Immunogenicity in mice**

Two groups, each containing five female BALB/c (60–90 days old) mice were immunized intraperitoneally with leaf extracts from transformed tobacco plants expressing H and nontransformed tobacco plants (10 µg of H per mouse) in complete Freund’s adjuvant for the first injection followed by two boosters with half of the protein amount on Days 7 and 14 in incomplete Freund’s adjuvant. Eleven animals were bled retro-orbitally every week until 10 weeks postimmunization and serum was collected and tested for the presence of specific antibodies to H by ELISA using
recombinant H as the antigen. Antibody titers are expressed as the log of the reciprocal of the highest serum dilution that gives OD readings twice the mean OD of serum from five mice immunized with nontransformed tobacco plant extract.

Hemagglutination inhibition

Hemagglutination inhibition test to detect specific anti- serum was done as described previously by Norrby (1962).

Virus neutralization test

In vitro virus neutralization (both homologous and cross- neutralization) was performed using Vero cells grown in MEM medium. Vaccine strain of RPV (RBOK strain) and vaccine strain of PPRV Nig 75/1 were grown on Vero cells and titrated employing the TCID50 method (Reed and Muench, 1938). Pooled serum samples (for each bleed, serum was pooled from all the five mice of the group) were heat-inactivated at 56 °C for 30 min followed by incubation with 100 TCID50 of virus at 37 °C for 1 h; 2 × 10^5 cells were added to each well. The wells without the sera/virus served as control. The plates were monitored for 5–7 days for cytopathic effects (CPE) for RPV and 3–5 days for PPRV. Virus neutralization titer was defined as the highest dilution of the sera, which inhibited CPE by 50%.

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