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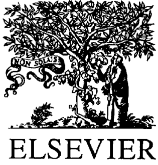

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Oral immunization of cattle with hemagglutinin protein of rinderpest virus expressed in transgenic peanut induces specific immune responses

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Abstract

Rinderpest is an acute, highly contagious often fatal disease of large and small ruminants, both domestic and wild. Global eradication of rinderpest needs a robust, safe and cost-effective vaccine. The causative agent, rinderpest virus (RPV) is an important member of the genus *Morbillivirus* in the *Paramyxoviridae* family. We have generated transgenic peanut (*Arachis hypogea* L.) plants expressing hemagglutinin protein of RPV and report here, the induction of immune responses in cattle following oral feeding with transgenic leaves expressing hemagglutinin protein without oral adjuvant. Hemagglutinin-specific antibody was detected in the serum as confirmed by immunohistochemical staining of virus-infected cells, and in vitro neutralization of virus infectivity. Oral delivery also resulted in cell-mediated immune responses.

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Keywords: Rinderpest virus; Hemagglutinin; Transgenic peanut; Oral immunogenicity

1. Introduction

Rinderpest is an acute, febrile, highly contagious disease of cattle caused by rinderpest virus (RPV), which is a member of the family *Paramyxoviridae* and genus *Morbillivirus*. In spite of availability of a highly effective live attenuated vaccine, rinderpest remains a threat to livestock in developing countries. The difficulty in maintaining the cold chain results in failure of vaccination in the hot regions where rinderpest is endemic. Attempts have been made to develop heat stable rinderpest vaccines, which include thermostable Vero cell-adapted rinderpest vaccine [1,2], Xerovac live attenuated rinderpest vaccine [3] and dry powder rinderpest vaccine [4]. RPV contains two glycoproteins, hemagglutinin (H) and fusion (F) proteins, on its host cell membrane-derived envelope. H and F proteins are known to be highly immunogenic and confer protective immunity. Efforts have been made to develop recombinant vaccinia virus expressing H and F [5–9] and recently long-term immunity in cattle has also been demonstrated [10,11]. Because of its wide host range, the use of recombinant vaccinia virus remains a matter of debate. Another pox virus (capripox virus), which has more restricted host range, has been used to develop recombinant capripox–rinderpest virus vaccine [12,13] and vaccination with this vaccine confers long-term immunity in African cattle [14]. Further, the H and F proteins expressed by recombinant baculo virus was shown to be immunogenic [15] and a recombinant baculo virus expressed H protein could induce both humoral and cell-mediated immune response [16,17]. In addition, the recombinant H expressed as extracellular baculo virus particles has been shown to elicit cytotoxic T-cell responses and a CTL epitope on H has been mapped [18,19]. Although the above-mentioned efforts promise to provide an effective vaccine, their use becomes prohibitively expensive because of the cost of production of cell culture vaccine.

In order to effectively control and eliminate rinderpest, a vaccine is necessary which provides a handle to differentiate between animals that have been vaccinated and those, which have recovered from natural infection [20]. For pathogens, which enter and colonize in the mucosal epithelium of gastrointestinal, respiratory and genital tract, it would be better to employ a mucosal vaccine since induction of both mucosal and systemic immune responses is achieved whereas the reverse does not hold true. A recombinant subunit vaccine expressed in edible parts of transgenic plants promises to possess the desired properties. In case of foot and mouth disease and transmissible gastroenteritis, recombinant antigens expressed in transgenic plants have been reported to possess immunological properties at least

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in the mouse model [21,22]. We have recently shown that the H protein of RPV expressed in a model plant, tobacco is immunogenic and elicit specific humoral immune response [23]. Further, we have expressed the hemagglutinin protein in transgenic peanut, a crop which is also used in India for animal feeding after harvesting the nuts, and tested its antigenicity and immunogenicity. This peanut-derived H is immunologically active when delivered through parenteral or through oral route in experimental mouse model system (Khandelwal et al., submitted for publication). In the present communication, we report the induction of specific immune responses in cattle upon oral feeding with transgenic leaves of peanut expressing H without mucosal adjuvant.

2. Materials and methods

2.1. Animals

Four cattle (C1–C4) were maintained at the Central Animal Facility of the Institute. C1, C2 and C4 (4–5 years age) of Holstein–Friesian cross-breed and C3 (about 2–3 years age) is a Jersey breed.

2.2. Cells and viruses

Vero cells were obtained from National Center for Cell Science, Pune, India and were maintained in MEM supplemented with 5% fetal calf serum (Gibco-BRL, USA) at 37 °C in a CO_2 incubator. A tissue culture adapted vaccine strain of RPV (RBOK) [24] was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India and vaccine strain of PPRV (Nig 75/1) was provided by Dr. A. Diallo, CIRAD-EMVT, France. To prepare infected cell lysates, at 24–48 h post-infection of Vero cells when 70% CPE was seen, cells were lysed in PBS by freezing and thawing three times and stored at −20 °C till further use.

2.3. Recombinant proteins

Recombinant hemagglutinin protein expressed in insect cells secreted into the medium (SecH) was used [25]. Recombinant nucleocapsid protein of RPV expressed in *E. coli* was purified by CsCl gradient as described earlier [26]. The full length M gene of RPV (RBOK) was cloned into pBluesript KS^+ vector (kindly provided by Dr. M. Baron, Institute for Animal Health, Pirbright, UK) was subcloned into pRSET expression vector and expressed in *E. coli* BL21 (DE3) (Shaji and Shaila, unpublished data), as His tag protein. The protein was purified on a nickel affinity column.

2.4. Antibodies

A mouse monoclonal antibody D2F4 to RPV H protein earlier generated in the laboratory [27] was used. Polyclonal monospecific antibodies to RPV H purified from infected cell extracts were generated in rabbits [28].

2.5. Transgenic peanut plants

The hemagglutinin gene of attenuated strain (RBOK) of rinderpest virus was subcloned into binary vector pBI 121. In the recombinant binary vector pBI H, the H gene is under the control of constitutively expressed CaMV 35S promoter. pBI H was mobilized into *Agrobacterium tumefaciens* (EHA 105). Transgenic peanut plants obtained using pBI 121 served as the control and termed as vector-transformed peanut plants. Transgenic peanut plants expressing hemagglutinin protein were generated via *Agrobacterium*-mediated transformation of shoot apices (Khandelwal et al., submitted for publication). Total protein from leaves was isolated employing the method of McGarvey et al. [29] used for the solubilization of rabies virus glycoprotein. The expression level of H was in the range of 0.2–1.3% of total soluble protein as estimated by double antibody sandwich ELISA using a standard curve where recombinant H was used as an antigen. Leaves from different lines expressing H were pooled and fed to the animals such that each dose contained an amount of H equivalent to 0.5% of total soluble protein.

2.6. Immunization schedule

The animals were fed with either transgenic peanut leaves expressing H (C2–C4) or with vector-transformed peanut leaves (C1) as control at weekly interval for three consecutive weeks. The first immunization was with about 7.5 g of leaves followed by 5 g of leaves at 7 and 14 days. Animals were given normal feed at all times.

2.7. Competition ELISA

The method described by Anderson and McKay [30] was used. The assay was performed in a 96-well flat bottom plate. SecH was used as the antigen (1:150 dilution in PBS) and D2F4 monoclonal antibody (1:5000) was used for competition with test serum. The reaction was developed with 50 μl of OPD (4 mg/ml) and H_2O_2 (2 μl of 30% stock) in PBS after terminating the reaction with 50 μl of 2N H_2SO_4, plate was read at 490 nm in an ELISA reader, which is attached to a computer having enzyme immunoassay (EIA) software of Biologicals Diagnostic mSupplies Ltd. (BDSL) and the OD values were converted to percentage inhibition (PI) values. Percentage inhibition more than 40 was considered to be significant.

2.8. Immunohistochemical staining

The method described by Naik et al. [16] was used to test the immune sera for reactivity with RPV H made in virus-infected cells.

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2.9. Virus neutralization test

Serum samples collected at various time points were tested for the presence of neutralizing antibodies (both homologous and cross-neutralizing) in triplicates using flat-bottomed 96-well plates as described by Barrett et al. [6]. Attenuated strain of RPV (RBOK strain) and vaccine strain of PPRV Nig 75/1 were grown on Vero cells and titrated employing $TCID_{50}$ method [31]. 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 and then double diluted with culture medium, starting from an initial dilution of 1:20. Following incubation with 100 $TCID_{50}$ of virus at 37 °C for 1 h, 2×10^4 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%.

2.10. In vitro lymphoproliferation

Animals were bled through jugular vein puncture at specified times. The blood was diluted 1:2 in sterile PBS and was subjected to Ficoll–Hypaque (Pharmacia) density centrifugation at 3000 rpm for 30 min. The buffy coat was collected and diluted in excess PBS. The cells were recovered by centrifugation and washed and resuspended in RPMI 1640 supplemented with 10% FBS (Gibco-BRL, USA). The lymphocytes were proliferated in triplicates at a density of 2×10^5 cells per well in presence of varying concentrations of SecH, N, M proteins or PPRV infected cell lysate or uninfected cell lysate in a final volume of 200 μl per well for 5 days. The cells were pulsed with 1 μCi [^{3}H]-thymidine (specific activity 6500 mCi/mmol; Amersham) for 16 h and harvested on glass fiber filter (Nunc, USA). The incorporated radioactivity was measured in a Rackbeta scintillation spectrometer.

3. Results

3.1. Humoral immune responses

Animals were bled at regular intervals after immunization either with transgenic peanut leaves expressing H or vector-transformed peanut leaves and sera were examined for the presence of H-specific serum antibodies in ELISA using recombinant secretory form of H (SecH) in a competition ELISA. Inhibition of binding to H protein of monoclonal antibody (D2F4) generated against RPV H by immune sera (Fig. 1) demonstrates the specificity of antibody. The serum from immunized animal, which received transgenic peanut expressing H competed very well with the monoclonal antibody and no significant competition was seen in presence of serum from the cattle fed with vector-transformed peanut leaves.

The specificity of the antibody produced in response to oral delivery of recombinant H as part of food was also verified by immunostaining of the infected cells. When antibodies from the orally fed animals were used for reactivity with antigens expressed in virus-infected cells only the immunized cattle serum reacted with viral antigen expressed in infected cells and the immune serum from the control animal that was fed with vector-transformed peanut leaves did not show any reactivity (Fig. 2) suggesting the capability of induced antibodies to recognize the antigens made by infected cells.

3.2. In vitro neutralization of virus infectivity

To analyze the in vitro protective ability of the induced antibodies, virus neutralization test was performed. Results (Tables 1 and 2) clearly show that high levels of neutralizing and cross-neutralizing antibodies are present in the serum 1 week after immunization and are maintained up to the duration of the experiments in the orally immunized animals. Homologous and heterologous virus neutraliz-

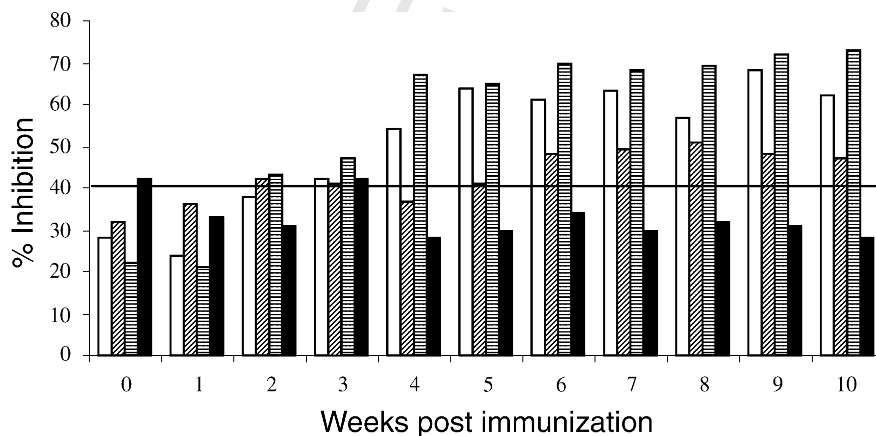

Fig. 1. Competition of immunized cattle serum with monoclonal antibody for recombinant H protein. Percent inhibition is calculated using the formula: PI = 100 − [(OD in test well/OD in 0% control well)] × 100. C1–C4 are represented as follows: C1 (■), C2 (□), C3 (▨) and C4 (▤).

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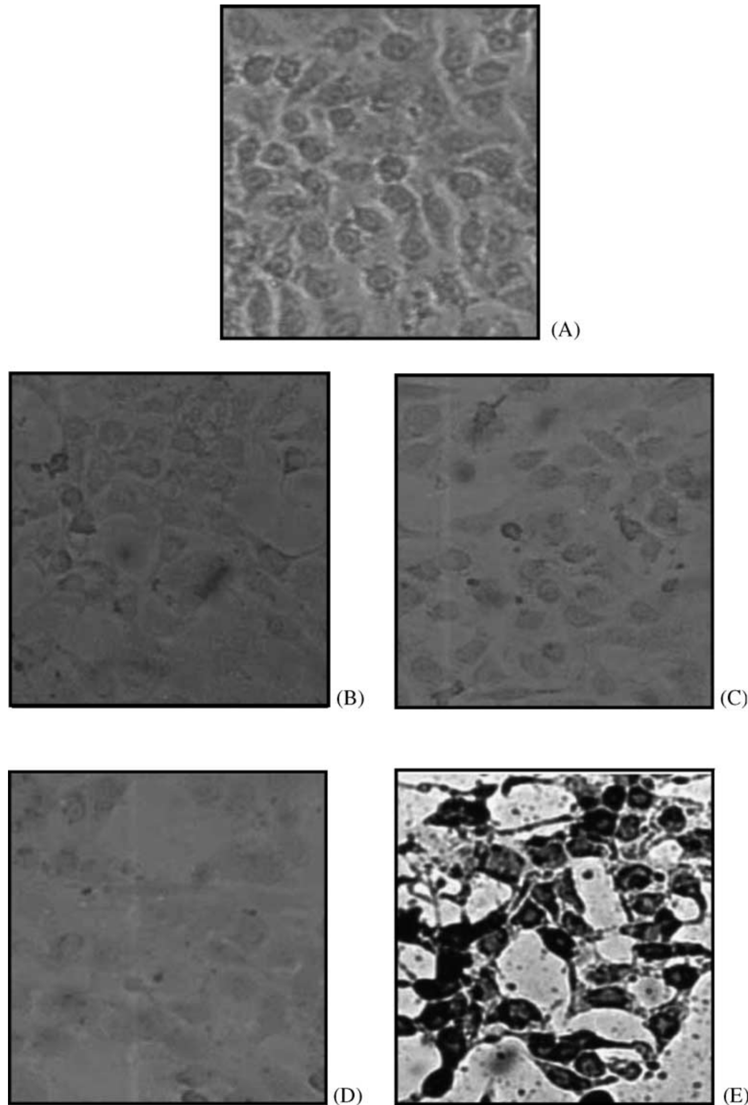

Fig. 2. Immunohistochemical staining. Panel A: uninfected Vero cells. Panels B and C: infected Vero cells immunostained with preimmune and immune serum (1:50 dilution in PBS) from animal fed with vector-transformed leaves (C1), respectively. Panels D and E: infected Vero cells stained with preimmune and immune serum (1:50 dilution in PBS) from animal fed with transgenic peanut leaves (C4), respectively. Vero cells grown on coverslips were infected with RPV (RBOK) at a multiplicity of 0.1–0.5 and incubated at 37 °C, for 48–72 h till 30–40% CPE was seen. Coverslips were washed in PBS and cells were fixed for immunohistochemical staining.

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231 ing ability was absent in serum from the animal fed with 232 vector-transformed peanut leaves. Earlier work from our 233 group on virus neutralization titer following vaccination 234 had shown virus neutralization titers of 20, 40, 40, 160 at 235 1, 2, 4 and 8 weeks post-immunization and nearly twofold 236 lower titers of cross-neutralization against PPRV [17]. How-237 ever, immunization of cattle with recombinant baculovirus 238 expressing H (rECV-H) resulted in a twofold higher titer 239 for both homologous and heterologous virus neutralization 240 titers. Induction of high levels of virus neutralization titer 241 immediately at 1 week post-immunization could be due 242 to the adjuvant activity provided by some plant compo-243 nent. These results demonstrate that antibodies generated 244 upon oral immunization are able to provide protection against RPV or cross-protection against PPRV infections 245 in vitro. 246

3.3. Lymphoproliferative responses 247

At the end of 10th week post-immunization, PBMC were 248 isolated and used for in vitro proliferation assay in pres-249 ence of recombinant antigens (Fig. 3A and B). PBMC 250 from animals fed with transgenic peanut leaves expressing 251 H proliferated in a dose-dependent manner when SecH was 252 used as the antigen (Fig. 3A), and the animal (C1) fed with 253 vector-transformed peanut leaves did not show any prolif-254 eration in response to SecH. The specificity of lymphopro-255 liferative responses was tested by stimulating the PBMC in 256

Table 1
In vitro neutralization of RPV infectivity by serum collected from cattle after oral immunization with transgenic peanut leaves or with vector-transformed peanut leaves

Animal	Virus neutralization titer[a] (days post-immunization)										
	0	7	14	21	28	35	42	49	56	63	70
C1	0	0	0	0	0	0	0	0	0	0	0
C2	0	320	320	640	80	40	40	40	40	40	20
C3	0	640	640	320	160	160	160	160	80	40	20
C4	0	160	320	320	160	160	160	40	40	40	40

[a] Virus neutralization titer is defined as the reciprocal of the highest dilution of serum exhibiting 50% protection of infected cells.

presence of other antigens of RPV. Data shown in Fig. 3B shows that lymphocytes from orally immunized animals do not respond to other antigens of RPV (nucleocapsid (N) protein and matrix (M) protein). Although the lymphocytes from vaccinated animal have been shown to proliferate in response to N protein of RPV [18]. In addition, when the cross-reactive proliferative responses to PPRV antigens were tested (Fig. 3C), lymphocytes from the animal immunized with peanut-derived H proliferated well in vitro in presence of PPRV infected cell lysate and the animal that received vector-transformed peanut leaves did not respond.

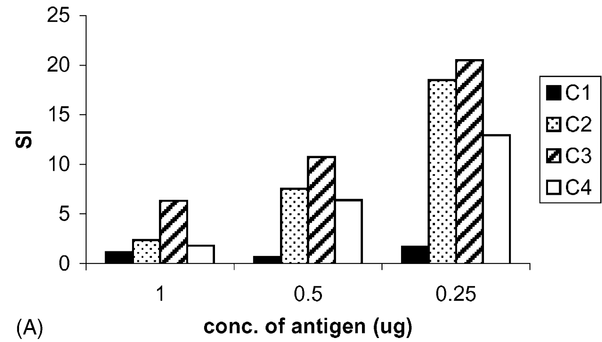
4. Discussion

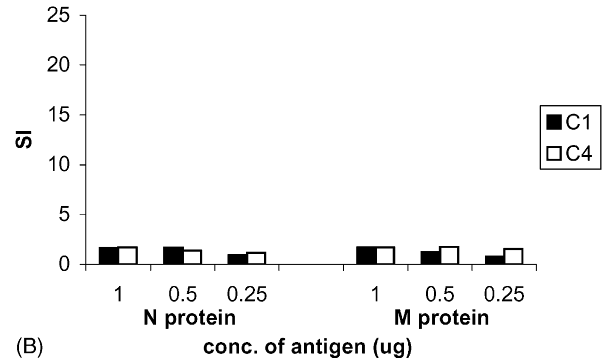
As part of efforts to develop edible vaccine for rinderpest, we generated transgenic peanut (*Arachis hypogea* L.) plants expressing hemagglutinin protein of rinderpest virus. The antigenicity of peanut-derived H protein was established using specific antibodies and its immunogenicity was analyzed in a mouse model (Khandelwal et al., submitted for publication). Oral feeding of transgenic peanut leaves induced specific mucosal (secretory IgA) and systemic immune responses (serum IgG and IgA) and also cell-mediated immune responses. In the present work, induction of immune responses in cattle was monitored upon oral delivery of hemagglutinin protein of rinderpest virus as part of food, without any mucosal adjuvant. To our knowledge, this is the first report describing elicitation of specific immune responses in the host animal by a protective antigen of a

Table 2
In vitro neutralization of PPRV infectivity by serum collected from cattle after oral immunization with transgenic peanut leaves or with vector-transformed peanut leaves

Animal	Virus neutralization titer[a] (days post-immunization)										
	0	7	14	21	28	35	42	49	56	63	70
C1	0	2	2	2	2	2	4	2	2	0	0
C2	0	128	256	256	256	64	32	32	32	32	8
C3	0	0	0	0	0	64	128	128	64	64	16
C4	0	128	256	256	64	64	64	32	32	16	8

[a] Virus neutralization titer is defined as the reciprocal of the highest dilution of serum exhibiting 50% protection of infected cells.





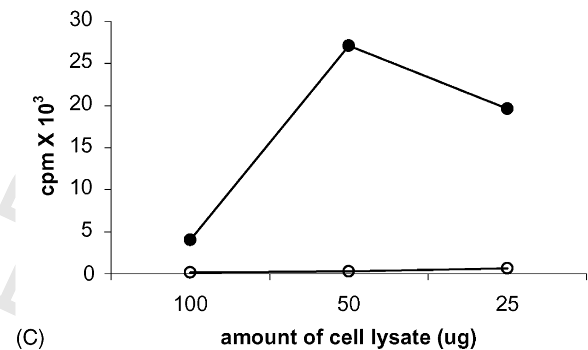


Fig. 3. (A) Lymphoproliferation of PBMC from animals fed with either vector-transformed peanut leaves (C1) or with transgenic peanut leaves (C2–C4) at 10 weeks post-immunization in presence of recombinant hemagglutinin protein. (B) Lymphoproliferation of PBMC from C1 (control animal fed with vector-transformed peanut leaves) and C4 (fed with transgenic peanut leaves expressing H) in presence of nucleocapsid (N) and matrix (M) protein of RPV. (C) In vitro proliferation of PBMC from C1 (○) and C4 (●) in presence of antigens of PPRV.

Morbillivirus expressed in transgenic plants given orally. Although small quantities of transgenic plant tissues (7.5 g for the first feeding followed by two feedings of 5 g) was given orally, the test animals developed high titer of specific antibodies. These antibodies were able to compete out monoclonal antibodies in ELISA (Fig. 1) demonstrating the specificity of the induced antibodies; in addition, these antibodies neutralized the virus infectivity in vitro. Animals were fed only thrice with plant-derived antigen at weekly intervals, which in addition to production of significant levels of specific antibody, resulted in stimulation of T cells from immunized animals in response to specific antigens

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(Fig. 3A and B) indicating the induction of systemic immune response upon oral immunization. Wigdorovitz et al. [21] reported induction of protective systemic immune response in the mouse model upon oral feeding of transgenic plants expressing VP1 protein of foot and mouth disease virus. In this work, the VP1 protein expressed in alfalfa plants was not detected by Western blotting and several immunizations (three times a week for 2 months with approximately 0.3 g of leaves) were needed in order to induce a significant immune response. Similarly, Gomez et al. [22] have shown oral immunogenicity of the spike protein of swine-transmissible gastroenteritis coronavirus expressed in potato in a mouse model. This group followed almost similar immunization schedule as reported by Wigdorovitz et al. [21]. However, there was no detectable neutralization activity, which was attributed to the post-translational processing in the host plant. Compared to these two reports, in the present work, small quantities of peanut expressed H protein given orally without adjuvant induced high levels of virus neutralizing antibodies.

There are two reports where induction of specific immune response is demonstrated upon oral feeding of human volunteers with potato tubers expressing LT-B of *E. coli* [32] or Norwalk virus capsid protein-assembled as virus like particles [33]. In the first human trials, the antigen used (LT-B) is a well-known mucosal adjuvant and therefore when given through oral route, LT-B antigen induced significant systemic and mucosal immune responses. In the second trial, potato expressing Norwalk virus capsid protein was delivered orally. It has been suggested that the particulate nature of the virus like particles confer greater stability to the antigen in the stomach and resulted in specific immune response although the level of specific serum antibody was modest. Induction of specific immune response in mice upon oral delivery of measles virus hemagglutinin expressed in plant tissues has been demonstrated [34]. The induction of immune responses upon oral delivery shown in the present work might be due to "bioencapsulation" as described by Kong et al. [35]. Modelska et al. [36] have shown that expressed antigen is more immunogenic when plant material is fed orally as compared to the plant proteins present in the extract. Additionally, there is evidence to suggest that components of the plant also influence the immunogenicity of the antigen expressed [22]. The induction of serum or mucosal antibody response to orally administered antigens is often difficult and generally requires large quantities of antigen as only part of the antigen is being absorbed and is capable of eliciting an immune response. It was assumed that co-administration of a mucosal adjuvant is necessary to achieve optimum oral immunogenicity for a given antigen. Furthermore, the presentation of large amount of antigen may lead to oral tolerance and use of mucosal adjuvant will result in non-specific stimulation of mucosal immune system. de Aizpurua and Russell-Jones [37] have identified the class of proteins that provoke an immune response upon oral feeding and concluded that all the proteins that possess "lectin or lectin-like" binding activities are active in oral immunization. These molecules have the ability to bind to glycolipids or glycoproteins on the intestinal mucosae and thus transported across the epithelial barrier, to enter the circulation and elicit an immune response. More recently, plant lectins with different sugar specificities have been investigated for mucosal immunogenicity [38] and elicitation of specific systemic and mucosal antibody response was observed upon intranasal or oral administration. Since hemagglutinin protein of RPV is a cell attachment protein which binds to cell surface oligosaccharide containing protein or glycoconjugate in order for the virus to begin the infection process, it qualifies to be in the categories of antigens having "lectin or lectin-like" activities. Therefore, it is conceivable that H protein may be transported across the epithelial barrier easily through the mechanism described by Lavelle et al. [38] and leading to systemic immune responses. It remains to be seen if this protein expressed in peanut plants elicits a mucosal immune response upon oral immunization.

Rinderpest is an economically important disease of livestock and certainly remains a threat to the world because of the isolated foci of the disease. History has witnessed the outbreak of the disease after 40 years of rinderpest-free Sri Lanka [39]. Since most part of the world is declared rinderpest-free, use of time tested live attenuated vaccine is restricted. And the infection cannot be diagnosed at an early stage since there is no simple test to differentiate between animals vaccinated with currently used vaccine and infected animals. The recombinant subunit oral vaccine expressed in plants is useful not only in differentiating vaccinated and infected animals but also offers a cost-effective means of mass vaccination by production of transgenic plants expressing the vaccine antigen in developing countries. In addition, it will have the advantage provided by an oral vaccine which results in induction of both mucosal and systemic immune responses better achieved through oral administration as compared to parenteral delivery of the antigen and may help in the first line of defense at the mucosal surfaces. Although we have not carried out any challenge experiments due to lack of high disease security and containment facilities, in vitro neutralization demonstrated the protective capability of the induced antibodies and priming of T cells, which are also involved in rinderpest immunity [15] and therefore the present work clearly demonstrates the potential of edible oral vaccine against rinderpest.

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