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Passive protection to bovine rotavirus (BRV) infection induced by a BRV VP8* produced in plants using a TMV-based vector

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Summary. We have previously reported on the use of a tobacco mosaic virus (TMV) vector TMV-30B to express foreign viral antigens for use as experimental immunogens. Here we describe the development of an improved TMV-30B vector that adds a sequence of 7 histidine residues to the C-terminus of recombinant proteins expressed in the vector. We used this TMV-30B-HISc vector to express the VP8* fragment of the VP4 protein from bovine rotavirus (BRV) strain C-486 in plants. Recombinant VP8* protein was purified from *N. benthamiana* leaves at 7 days post-inoculation by immobilized metal affinity chromatography. The plant-produced VP8* was initially detected using anti-His tag mAb and its antigenic nature was confirmed using both monoclonal and polyclonal specific antisera directed against BRV. Adult female mice, inoculated by the intraperinoteal route with an immunogen containing 4 μg of recombinant VP8*, developed a specific and sustained response to the native VP8* from the homologous BRV. Eighty five percent of suckling mice from immunized dams that were challenged with the homologous virus at the fifth day of age were protected from virus as compared to 35% of the pups from mothers immunized with a control protein. These results

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demonstrate that the plant-produced VP8* was able to induce passive protection in the new born through the immunization of dams. This suggests that the technology presented here provides a simple method for using plants as an inexpensive alternative source for production of recombinant anti-rotavirus antigens.

Introduction

Rotavirus is the principal etiological agent of severe neonatal gastro-enteritis in mammalian species [9], including infants and young children worldwide [1]. It is also responsible for extensive economic problems in animal species. In particular, bovine rotavirus (BRV) is responsible for approximately 5% of severe diarrhea in calves born in North America causing up to 7 billion dollars in losses to the cattle industry annually [16].

Rotavirus possesses a genome composed of 11 segments of double-stranded RNA contained in an inner core and surrounded by two protein layers that form the viral capsid. Virus particles present spikes formed by homo-dimers of a non-glycosylated protein, VP4, which pass through and extend from the virion surface layer [2].

This protein can be cleaved after trypsin digestion in to two smaller polypeptides of 65 and 28 kDa, termed VP5* and VP8*, respectively. This cleavage, which occurs during natural infection, strongly enhances rotavirus infectivity and virus penetration of cells [2].

Calves are susceptible to rotavirus infection within the first three weeks of life and for this reason, active immunization prior to exposure to virulent virus is almost impossible to accomplish. However, calves can be passively immunized by vaccination of the dam in order to increase their colostral antibodies, which are then transferred to the calves [19]. Similarly, in the mouse model, passive transfer of antibodies from immunized dams to suckling mice can also prevent rotavirus infection [13].

Previous reports have identified several advantages of the VP8* subunit as an immunogen. Antibodies directed to the VP8 fragment, were capable of neutralize infection *in vitro* by preventing the virus binding to cells [17]. In addition, parenteral vaccination with a recombinant VP8* produced in *E. coli* was able to: (i) boost BRV-specific neutralizing antibodies in mice and cattle pre-immunized with BRV inactivated particles [10], ii) elicit colostral antibody titres equivalent to those obtained with a commercial vaccine in pregnant cattle [25] and (iii) induce homotypic protection against disease in mice born from dams immunized with this antigen [4].

Plants represent a potentially promising source of biomass for economical production of recombinant biological products [5]. Transgenic plants have been frequently used to express immunorelevant antigens for use in the formulation of experimental vaccines for humans and animals [11]. Another practical alternative for high level expression of foreign proteins in plants is to transiently express the gene of interest from a recombinant plant virus vector [20]. Expression vectors like those derived from RNA plant viruses such as tobacco mosaic virus (TMV) have

had some significant applications in commercial biotechnology [3, 22, 27]. In this report, we describe the production of a recombinant VP8* from BRV strain C486 (P[1]G6) in plants utilizing a modified version of the TMV-30B vector [21] that we had used previously for expression of vaccine antigens from foot and mouth disease virus (FMDV) [23] and bovine herpes virus-1 (BHV-1) [15]. We report here on a modification of the vector to permit insertion of a sequence of histidine residues (7 × HIS) at the C-terminus of the foreign insert in order to permit facile purification by immobilized metal affinity chromatography (IMAC) [12]. In the present study we show the expression of a recombinant VP8* from BRV in plants using this modified vector (TMV-30B-HISc). Our results demonstrate that the recombinant VP8* could be readily purified from plants, retained native antigenic and immunogenic properties and proved effective at inducing passive immune protection to the homologous challenge with virulent BRV in an experimental murine model.

Materials and methods

Plasmid constructions

The VP8* open reading frame (ORF) cDNA was obtained by reverse transcription and PCR amplification from RNA isolated from purified bovine rotavirus strain C486 (G6P1 serotype). The direct primer contained the *Pac*I site located upstream of the 5′ end of VP8* coding sequence and reverse primer contained, from 3′ to 5′, the *Pme*I restriction sequence and the 7 × HIS in frame with the 3′ end of the VP8* ORF. The resulting 783 bp PCR product was digested with *Pac*I and *Pme*I restriction enzymes and cloned into the TMV-30B vector [21] using the same sites of vector polylinker to produce the TMV-VP8*HISc plasmid (Fig. 1). A second recombinant vector, TMV-p24HISc, was produced following

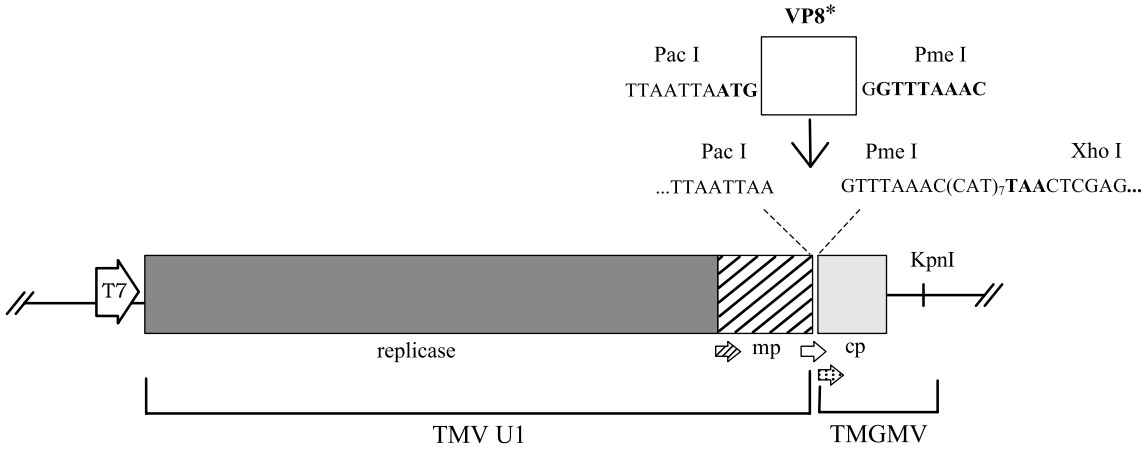

Fig. 1. Schematic of the TMV-30B-VP8* vector. Location of the TMV-derived genes in the vector and the VP8* insert located between the *Pac*I and *Pme*I sites in the polylinker sequence. Relevant restriction sites as well as the additional histidine sequence [$(CAT)_7$)] and stop codon, are also indicated. Subgenomic promoters are indicated by small arrows below the movement protein (*mp*), coat protein (*cp*) and inserted foreign ORF. *In vitro* transcription was driven from the T7 promoter located upstream the 5′ end of the TMV U1 replicase gene

a similar strategy as for the TMV-VP8*HISc. This vector, carrying the core protein (p24) from the human immunodeficiency virus-1 (HIV-1; Perez Filgueira et al., manuscript in preparation), was utilized for production of the p24-HIS protein, which served as control for the immunological experiments. Integrity and correct framing for both ORFs were confirmed by DNA sequencing.

Plant inoculation

The TMV-VP8*HISc and TMV-p24HISc plasmids were digested with *Kpn*I to generate templates for *in vitro* production of capped RNA transcripts (Amplicap T7 transcription Kit, Epicentre Technology). Infective viral RNAs were quantified and checked for integrity by electrophoresis before being applied to the upper surface of *N. benthamiana* leaves as previously described [15]. Plants were kept in growth chambers (16 hrs/day of light, 29 °C day temperature, 24 °C night temperature) and leaves were harvested 7 days post-inoculation (dpi) and frozen at −20 °C until processed.

Production and purification of plant recombinant proteins

Plant sap was obtained by grinding inoculated plant leaves in extraction buffer containing phosphate buffered saline (PBS) and 8 M urea at pH 8.0. Extracts were filtered through Miracloth (Calbiochem) and clarified by centrifugation at 10 K RCF for 10 min. IMAC resin (Talon, Clontech) was washed and resuspended in the same extraction buffer and incubated with clarified plant sap for 1 h at room temperature (RT). Beads were then washed 3 times with PBS/Urea 8 M pH 6.3 and placed into plastic columns. His-tagged proteins were separated from the IMAC resin with an elution buffer of 6 M Urea/300 mM NaCl/50 mM Na Acetate, using a pH gradient from pH 5.8 to 4.8.

Protein analysis

Eluted protein or IMAC resin samples were analyzed in 12% SDS-PAGE gels and either stained with Coomassie blue or transferred onto ECL Nitrocellulose membranes (Amersham). For Western-blot assays, membranes were blocked overnight at 4 °C with PBS-Tween 20 (PBST) 4% skim milk. Blots were subsequently incubated for 1 h at RT with either anti-6 × His monoclonal antibody (mAb) (Clontech) or one of the specific antibodies. This was followed by a mouse Ig-specific HRP-labeled conjugate as secondary antibody. Both primary and secondary antibodies were diluted in the same blocking buffer. Membranes were developed with X-film (Kodak) using the ECL Western blotting detection system (Amersham). Concentrations of the recombinant proteins were estimated from Coomassie blue-stained gels in a Biorad Fluor S MultiImager System using the Quantity One 4.0 software (BioRad).

Production of bovine rotavirus antigen

The C486 BRV strain was replicated in MA104 cells. Tissue culture supernatants were concentrated and semi-purified through a 40% sucrose cushion as described previously [26]. This VP4 enriched preparation was used as antigen for Western blot and ELISA [14].

Analysis of the immune sera

Western blot: Proteins from semi-purified C486 BRV were resolved in 12.5% SDS PAGE and gels were transferred onto nitrocellulose membranes (Immobilon P, Millipore). Blots were blocked overnight as described above and incubated for 1 h at RT with sera from mice immunized with the recombinant VP8*-HIS or p24-HIS. After washing with PBST, membranes were incubated with mice Ig-specific AP-labeled conjugate (KPL) and developed using NBT/BCIP (BioRad).

ELISA: Analysis was performed using 1 μg/well of semipurified C486 BRV as antigen. Mock-infected cultures were processed also and used to coat wells as a background control. Immunolon II plates (Dynatech) were coated overnight with the antigen diluted in 0.1 M carbonate–bicarbonate buffer pH 9.6 at 4 °C. Plates were then blocked with PBS containing 1% BSA (blocking buffer) and 0.05% Tween 20 (PBST) for 30 m at 37 °C. Plates were then incubated for 1 h at 37 °C with four-fold dilutions of mice serum samples, starting in a 1:20 and a secondary anti mouse-Ig specific HRP-conjugated antibody (1/4000) (KPL). Primary and secondary antibodies were diluted with the same blocking buffer. The reaction was developed by addition of 1 mg/ml of ABTS [2,2′-amino-di-(3-ethylbenzthiazoline sulphonate)] – 0.015% H_2O_2 in 1 M citrate buffer pH 5 and read 10 minutes later at 405 nm in a MR 500 Microplate Reader (Dynatech). Titers were expressed as log10 of the reciprocal of the highest serum dilution that gave a corrected optical density reading (OD antigen–OD mock) at least twice that of a pool of sera from non-immunized mice at the same dilution.

Immunization of animals

Eight weeks-old female Balb/c mice, previously tested for presence of rotavirus-specific antibodies using the ELISA test described above, were used in the experiments. Rotavirus seronegative mice were immunized 3 times with 0.3 ml of an emulsion prepared with incomplete Freund adjuvant and containing 4 μg of the recombinant VP8*-HIS or p24-HIS proteins in IMAC elution buffer diluted 1:4 in PBS. Preparations were administered by the intraperitoneal route (ip) every 2 weeks. Positive control sera included rabbit polyclonal hyperimmune serum against VP8* and a C486 BRV polyclonal antiserum raised in mice.

Challenge of neonates

Pups born to dams immunized with VP8*-HIS or p24-HIS one to three weeks before parturition were allowed to suckle their dams during the entire experiment. Suckling mice were orally challenged at the fifth day of age with 10^4 plaque-forming units (PFU) of virulent C486 BRV by intubation of the stomach with a flexible plastic tube. This dose of virus caused diarrhea in 100% of mice born from non-immunized dams [7, 8]. The presence of diarrhea in the challenged mice was monitored for 24 h and determined by the appearance of liquid stools upon gentle abdominal palpation.

Results

Expression of BRV VP8 using the TMV30B-HISc vector*

The TMV 30B vector was modified to permit incorporation of 7 histidine residues at the C-terminus of two viral proteins (see Fig. 1). TMV-VP8*HISc was created to evaluate the production of usable rotavirus antigen in plants and TMV-p24HISc was created to produce the HIV p24 protein which was use here as a control. *N. benthamiana* leaves were inoculated with RNA derived from each of the vectors and tissue was harvested at 7 dpi. The recombinant p24-HIS and VP8*-HIS proteins were isolated from freshly extracted plant sap by incubation with the IMAC resin followed by washing and elution prior to SDS PAGE and Western blot analysis. As previously observed (Perez Filgueira et al., manuscript in preparation), two stained protein bands were detected in extracts of leaf samples inoculated with the TMV-p24HISc vector (Fig. 2A, lane 2). A prominent band with an apparent molecular weight of 25 kDa and a minor band of about 50 kDa that was likely a dimer of the p24-HIS. Two poorly resolved bands of expected size for the

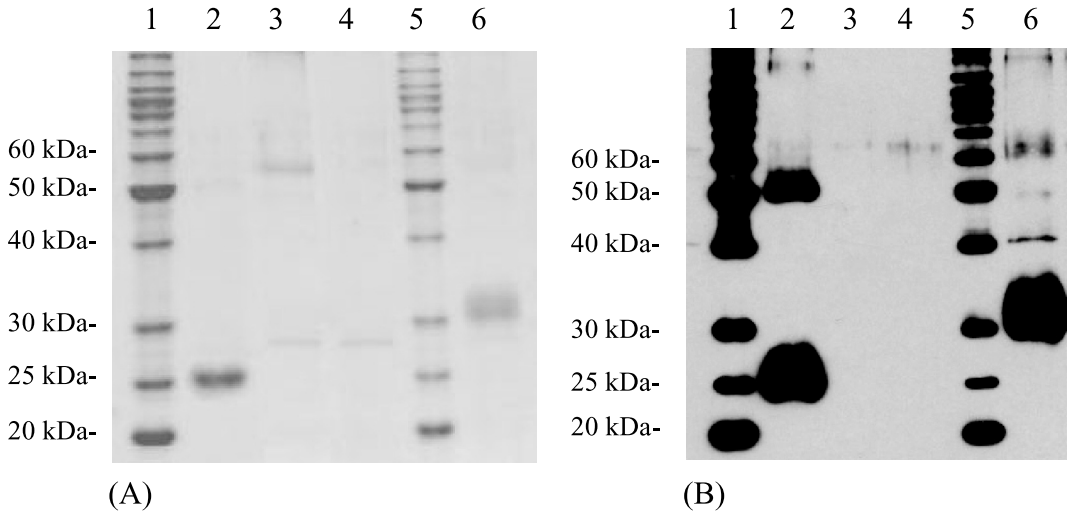

Fig. 2. Detection of recombinant proteins in plants inoculated with the TMV-30B-HISc vector. **A** Coomassie blue stained SDS-PAGE gel. **B** Western blot from the same gel probed with an anti-6 × HIS mAb. Both panels: *2–4* and *6*, samples of IMAC beads after incubation with the plant sap extracted from approximately 25 mg of inoculated leaf tissue: *4* mock; *2* TMV-p24HISc; *3* TMV-30B; *6* TMV-VP8*HISc; *1* and *5* HIS-tagged MWM

recombinant product (ca. 31 and 33 kDa) were consistently present in extracts of the TMV-VP8*HISc inoculated leaves (Fig. 2A, lane 6). We suspect the duplex was likely the result some proteolytic processing of the recombinant protein. A few minor contaminant proteins were normally detected in extracts from control mock and TMV-30B infected leaf samples (Fig. 2A, lanes 3 and 4). The HIS-tagged recombinant proteins were readily detected and easily distinguished from minor contaminants in Western blots developed using an anti-6 × HIS mAb (Fig. 2B, lanes 2 and 6). These results clearly demonstrate that the HIS tagged vector provided a very facile means for both isolating and unambiguously identifying both recombinant proteins in the plant extracts.

Antigenic characterization of the recombinant VP8-HIS produced in plants*

N. benthamiana plants were inoculated with the TMV-VP8*HISc vector and approximately 50 g of fresh leaf tissue was processed by IMAC to yield about 5 μg of recombinant VP8* protein per gram of fresh leaf tissue. We next wanted to confirm that the antigenic nature of the recombinant VP8* protein was preserved by synthesis in the plant.

i) *Western blot assays*: The VP8*-HIS recombinant protein isolated by IMAC was blotted onto nitrocellulose membranes and analyzed by Western blot using both BRV-specific monoclonal and polyclonal sera and a mAb against the HIS-tag (Fig. 3). The results showed that the same recombinant protein detected by the anti-6 × His mAb (Fig. 3A), also reacted positively to a polyclonal mouse serum

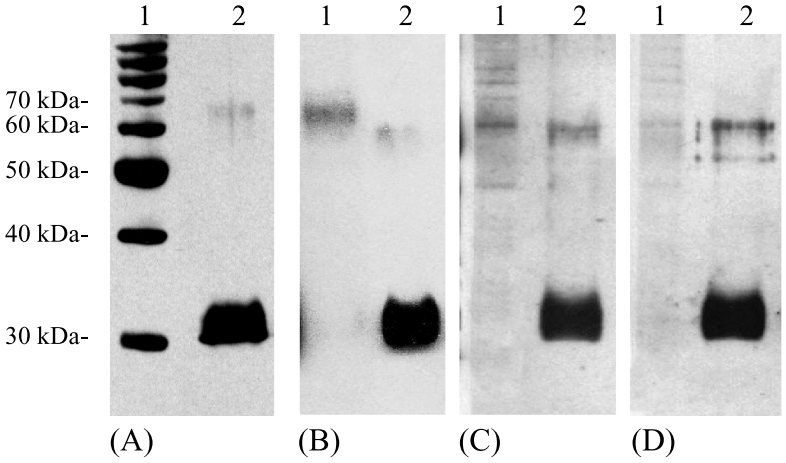

Fig. 3. Antigenic characterization of the VP8*-HIS protein produced in plants. Western blots were probed with: **A**) anti-6 × HIS mAb; **B**) BRV polyclonal serum against the p235–252; **C**) mAbs 2C10 and **D**) MAbs 1A8. For each panel, *1* contains the HIS-tagged MWM and *2* contains the VP8*-HIS protein

produced against the VP4 cleavage peptide, p232–255 [6] (Fig. 3B) as well as to both of the BRV-specific mAbs tested, 2C10 (Fig. 3C) and 1A8 (Fig. 3D) [18].

ii) *Analysis of the antibodies elicited by the recombinant VP8*-HIS*: Adult female mice were injected with purified VP8*-HIS and production BRV specific antibodies was analyzed by Western blot and ELISA. Control animals were injected with an equivalent amount of p24-HIS produced following the same procedure described for VP8*. Proteins from purified BRV were resolved by 12% SDS PAGE, transferred to nitrocellulose membranes and Western blots were performed using the immune sera obtained from the mice immunized with both the recombinant VP8*-HIS and p24-HIS proteins (Fig. 4A). Results demonstrated that while mice injected with the plant-produced VP8* antigen developed a specific humoral response against BRV (Fig. 4A, samples 1–5), animals inoculated with the control protein p24-HIS failed to induce BRV antibodies (Fig. 4A, sample 6). These results were further confirmed by testing the same serum samples for their reactivity to BRV particles in ELISA. The results in Fig. 4B show that all VP8*-HIS-immunized mice antisera presented antibodies that reacted positively against the rotavirus-derived antigens in their native form. As expected, the pool of sera from p24-HIS inoculated animals did not recognized the BRV particles. Additionally, immune and control sera did not show significant reaction in wells coated with the control antigens derived from mock infected cell culture supernatants (data not shown).

VP8-HIS produced in TMV infected plants induce a protective immune response*

To test the antigenicity of the plant-produced VP8*-HIS using an *in vivo* model, a challenge experiment was performed in passively immunized suckling mice. A

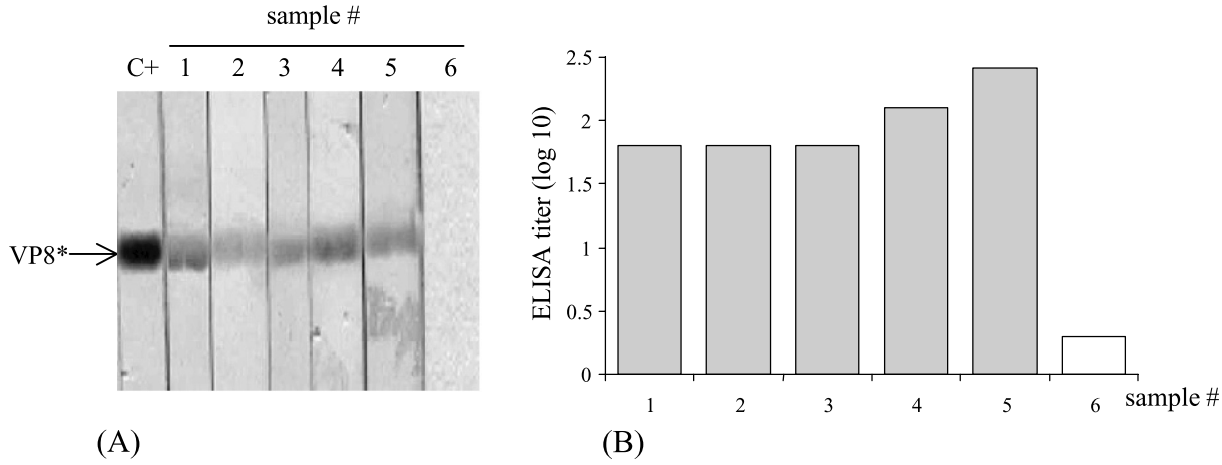

Fig. 4. Humoral response induced in mice by the VP8*-HIS protein. Primary inoculation and subsequent booster injections were performed using 4 μg of recombinant protein. Serum samples were extracted from mice 1 week after the second booster. **A**: Western blots against semipurified BRV. *2–7* show individual serum samples taken at 63 days after initial immunization of mice with VP8*-HIS (samples #1–5); *1* is the polyclonal anti-VP8 positive control; *8* is the pool of negative control sera from 5 animals immunized with the p24-HIS protein (sample #6). **B**: ELISA test of same serum samples (#1 to #6) tested against whole BRV particles as antigen. Titers are expressed relative to those of the control non-immunized animals as described in Materials and methods

second group of female mice were immunized with either recombinant VP8*-HIS or p24-HIS protein, and paired after the second immunization. Dams were allowed to produce litters which averaged about 5 new born mice each. The pups were born between 1 and 3 weeks after their dam's last booster. Sera from immunized dams and their corresponding pups were initially analyzed by Western-blot as

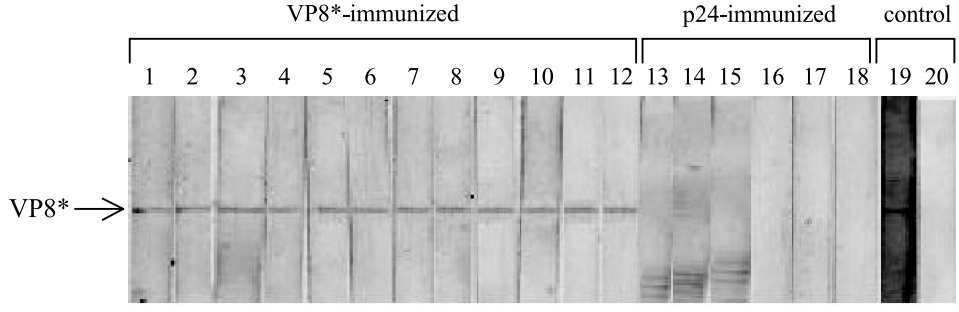

Fig. 5. Passively acquired humoral response in suckling mice induced by VP8*-HIS protein immunization of pregnant dams. Western blot of sera from dams immunized with VP8-HIS or p24-HIS and their pups reacting against a VP4-enriched BRV preparation. *1*, *4*, *7*, *10* developed with sera from dams immunized with VP8*-HIS; *2–3*, *5–6*, *8–9* and *11–12* developed with sera from their respective newborn progeny; *13*, *16* developed with sera from dams immunized with p24-HIS; and *14–15* and *17–18* developed with sera from their respective newborn mice. *19* is a positive control using BRV-specific polyclonal sera against the p235–252 and *20* is a pool of normal mice sera

Table 1. Challenge of suckling mice born from immunized dams

Dams immunized with	# Suckling mice protected/challenged (percentage of protection)	Fisher's Exact Test
VP8*-HIS	23/27 (85%)	$p<0.0004$
P24-HIS	8/23 (35%)	

Dams were immunized as described in Fig. 4. Litters were born 1 to 3 weeks after the last immunization. Newborn mice were challenged 5 days after birth with 100 µl of virulent BRV C486 (infectious titer of 10^5 PFU/ml) and observed for the presence of diarrhea for the next 24 h

above, revealing the existence of BRV-specific serum antibodies only in newborn mice from VP8*-HIS immunized dams and not in the control p24 immunized animals (Fig. 5). Litters were challenged with the homologous BRV, and as it is shown in Table 1, 85% of the suckling mice born from dams immunized with the plant-produced VP8*-HIS did not become infected when challenged with homologous virus 5 days after birth. In contrast, only 35% of the mice born from p24-HIS vaccinated dams did not become infected after challenge inoculation. These differences were statistically significant when analyzed by the Fisher's Exact Test indicating that passive protection induced by VP8*-HIS was significantly higher than that induced by the control p24-HIS injections ($p<0.0004$).

Discussion

We have described here the use of a recently developed TMV-based vector, useful for the production of the VP8* fragment of the bovine rotavirus structural protein VP4 in plants. We have shown that the VP8* fragment is expressed in the plants during virus infection and that it could be efficiently one-step purified by an affinity chromatography using a small His-tag artificially added to the carboxyl end of the protein. Finally, we also demonstrated that the purified recombinant VP8* was immunologically capable of eliciting a viral specific antibody response in vaccinated dams and it was able induce passive protection in their offspring.

We consider this application a simple but significant improvement over our previous use of the TMV-30B vector to produce FMDV and BHV-1 antigens in plants for potential vaccinal use [15, 23]. The evident advantages of altering the vector to add a sequence of 7 histidine residues to the end of the viral antigen include: i) the possibility to quantify the product following standard gel staining procedures, and ii) the capacity to simple detect and unambiguously identify the recombinant proteins using a commercially available mAb against the HIS-tag. This is a particular advantage for antigens for which specific antisera are not readily available. The results presented here demonstrate that two very different viral proteins, VP8*-HIS and p24-HIS, could be expressed at high concentration in a sufficiently solubilized form to be selectively bound to the IMAC resin with minimal levels of non-specific binding of other unrelated contaminant proteins.

The immunological characterization results using different BRV-specific antisera and mAbs, confirmed the antigenic integrity of the HIS-tagged proteins purified from leaves inoculated with the TMV-VP8*-HIS vector. All the VP8* specific antibodies tested, recognized the same denatured VP8*-HIS proteins, indicating that at least the epitopes reactive to these polyclonal sera and mAbs were preserved.

The animal immunization experiments demonstrated that the humoral response elicited by the plant-produced VP8* was capable of recognizing purified BRV C486 particles in denaturing Western blot assays as well as in their native form using ELISA tests. These last results indicated that the urea treatment did not significantly alter the antigenic structure of the protein, in accordance with previous publications that showed that urea concentrations as high as 10 M, did not affect binding and haemagglutination activities in the reovirus σ1 protein [24] and BRV VP8* [10]. Importantly, our results were unambiguous even when the amount of recombinant protein utilized for mice immunization was 20 to 25 times lower than that used in previous reports [10]. This positive humoral response was also consistent with the significantly higher rate of passive protection against virulent C486 challenge observed in pups born to VP8* immunized dams. Although there was a small percentage of unprotected mice, it is possible that if we had used higher doses of the VP8*-HIS, we may have been able to induce even higher protection percentages in the suckling mice.

In conclusion, we have shown that the BRV-derived VP8* antigen can be readily produced in plants inoculated with the recombinant TMV-VP8*-HISc vector. Our results suggest that this approach offers a very facile and economic means for producing inexpensive animal viral antigens in plants. Immunological characterization as well as challenge experiments also demonstrated that the plant-produced VP8*-HIS retained native antigenic properties and the ability to induce passive protection in a murine model. We think that these results demonstrate that this technology may have very real promise for practical application in the economic production of vaccinal antigens in plants for use in control of animal viral diseases such as Bovine rotavirus.

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