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# Bovine herpes virus gD protein produced in plants using a recombinant tobacco mosaic virus (TMV) vector possesses authentic antigenicity

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**Abstract**

A tobacco mosaic virus (TMV)-based vector was utilized for expression of a cytosolic form of the bovine herpesvirus type 1 (BHV-1) protein glycoprotein D (gDc). *Nicotiana benthamiana* plants were harvested 7 days after inoculation with RNA transcripts derived from the TMV-gDc recombinant virus. Recombinant gDc protein of expected electrophoretic mobility accumulated in inoculated leaves to a concentration of about 20 μg/g of fresh leaf tissue. Oil-based vaccines were formulated with crude foliar extracts to immunize mice parentally. After a single injection, animals developed a sustained and specific response to both the isolated gD and native virus particles. Cattle vaccinated with the same gDc containing extracts developed specific humoral and cellular immune responses directed against both the viral gD and BHV-1 particles. Most importantly, animals vaccinated with the plant-produced gDc showed good levels of protection after challenge with the virulent BHV-1. Virus excretion was drastically reduced in these animals, reaching levels comparable to animals vaccinated with a commercial BHV-1 vaccine. The positive immunological characterization obtained for the gDc, indicated that an important part of the natural conformation was retained in the plant recombinant protein.
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*Keywords:* TMV-based vectors; Recombinant protein; Bovine herpes virus-1 (BHV-1)

## 1. Introduction

Plants represent one of the most abundant biomass sources for large-scale production of biological products. Several reviews on the application of this technology argue that it offers cost efficiency over more expensive fermentation or cell culture based methods [1–3]. Particularly in the vaccine field, several publications reported the development of transgenic plants expressing successful immunogens for oral or parenteral immunization [4–7]. Foreign proteins can also be produced in plants by transient expression using plant virus-based vectors [8]. The ability of these vectors to achieve transient expression of a foreign gene product has some advantages over constitutive expression from a transgenic plant. Most notable is the fact that products of interest can be produced very rapidly after inoculation of mature plants with the additional benefit that the recombinant proteins can accumulate to high levels without appreciable degradation or toxic effects on the plant. Plant viral-based vectors have been successfully produced from a variety of plant RNA viruses and can be generally classified in those that can display foreign peptides fused to the coat proteins on the surface of viral particles [9] and those that are able to express whole recombinant proteins that accumulate within the plant, independently from other vector proteins [10]. Tobacco mosaic virus (TMV)-based expression vectors represent one of the more successful examples of this technology [11] and have been utilized for production of

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different therapeutic proteins, such as α-trichosanthin [12] and tumor-specific single-chain antibodies [13]; and vaccine antigens, such as a malarial peptide [14] and the VP1 structural protein from foot-and-mouth disease virus (FMDV) [15]. We report here the use of the TMV-30B vector [16]—which typifies a relatively stable family of TMV-based protein expression vectors—for the production in plants of a protein from bovine herpesvirus type 1 (BHV-1).

BHV-1 is the causative agent of a group of respiratory and reproductive disorders in cattle that is commonly referred to as infectious bovine rhinotracheitis [17]. The disease affects both adult and young animals and is of significant economic importance worldwide. Usual symptoms include rhinotracheitis, infectious pustular vulvovaginitis, enteritis, general respiratory disease and encephalitis, decreased milk production, weight loss and abortion in pregnant cows [18]. The BHV-1 vaccines in current use are formulated with either inactivated or modified live virus and both have a number of disadvantages. The inactivated vaccines are often poor immunogens and they have been shown to cause clinical disease if they are insufficiently inactivated [19]. Alternatively, live vaccines may induce immunosuppression [20] and complicate the process of discerning between vaccinated and infected animals. Alternative vaccination strategies involving the use of individual viral components such as glycoprotein D (gD) to induce protective immune responses to BHV-1 have been explored to overcome these problems [21–24].

In this report, the TMV-30B vector was utilized to produce a truncated form of the BHV-1 gD (cytosolic or gDc) protein in plants. Crude extracts of leaves inoculated with the recombinant virus were used to parentally immunize mice and cattle. We were able to show that these plant extracts induced both humoral and cellular-specific responses that recognized both the isolated protein and BHV-1 virus particles. Our results also demonstrated that the recombinant gDc could be stably produced in plants and that it retained antigenic integrity. More importantly, we showed that crude plant extracts containing the gDc protein had sufficient immunogenic potential to induce protection in the natural host against viral challenge.

## 2. Materials and methods

### 2.1. Construction of the TMV-based expression vector carrying BHV-1 gDc

The recombinant TMV-gDc vector was obtained from a variant of the TMV-30B expression vector [16] kindly provided by Dr. W.O. Dawson. Cloning of the foreign sequence into the TMV vector was as previously described [15]. The gDc sequence corresponding to nucleotides 54 through 1079 of the full-length gD was obtained by PCR from a pGem plasmid carrying the BHV-1 *gIV* gene. The primers, gDc-*Pac*I, (5′-ATTAATTAATGAGCTTG-CCTACACCCGCGCCG-3′) and gDc-*Pme*I (5′-GTTTAAA-CTCAGGCGTCGGGG GCCGCGGG-3′), were used to introduced start and stop codons at the extremes of the gDc sequence along with *Pac*I and *Pme*I restriction sites, respectively. The resulting fragment was then digested with *Pac*I and *Pme*I and inserted in the polylinker sequence of the TMV vector (Fig. 1). Integrity and correct insertion of the gDc sequence into the TMV-30b vector were confirmed by restriction analysis and nucleotide sequencing. The TMV-gDc plasmid was linearized by digestion with *Pst*I and used as DNA template for the in vitro production of the infectious viral capped RNA following manufacturer instructions (Amplicap T7 transcription Kit, Epicentre Technology). *Nicotiana benthamiana* plants were mechanically inoculated on the upper surface of mature leaves using infectious RNA transcripts diluted in buffer containing 1% Na-pyrophosphate (pH: 9.0), 1% celite and 1% bentonite. Plants were kept in growth chambers (16 h per day of light, 30 °C day temperature, 24 °C night temperature) until harvested. Inoculated foliar material was stored at −20 °C until utilized.

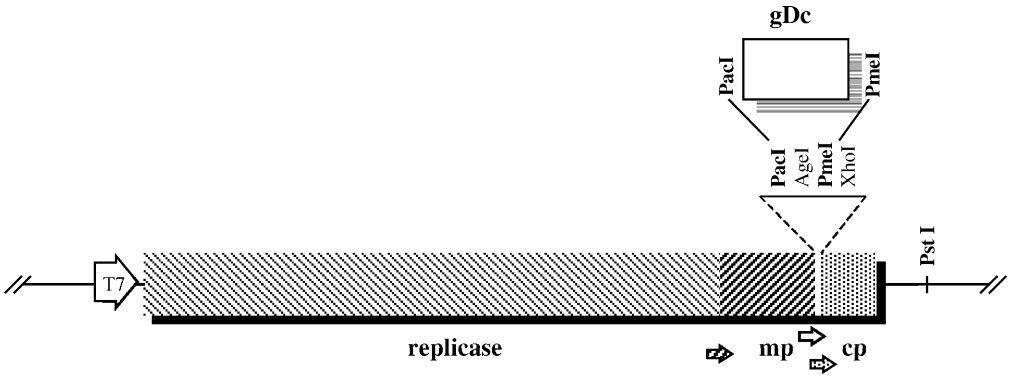


Fig. 1. Schematic structure of the TMV-gDc vector. The figure shows the location of the TMV-derived genes in the vector (the RNA-dependent RNA polymerase (replicase), the movement protein (mp) and the coat protein (cp)) and the gDc insert (1.05 kb) located between the *Pac*I and *Pme*I sites in the polylinker sequence. Subgenomic promoters are indicated as arrows upstream of the corresponding genes as is the location of the T7 promoter and key restriction sites.

### 2.2. Western blot analysis of the TMV-gDc infected leaves

Western blot analyses were performed on extracts from inoculated leaves using a polyclonal mouse antiserum specific for a recombinant gD produced in *Eschirichia coli* (*gD-Ec*) [25]. Leaves were harvested at 7 days post-inoculation (dpi) and total protein was obtained from inoculated leaves by an organic extraction protocol following the manufacturer instructions (Tripure, Roche). The extracted proteins were separated in a 12.5% SDS polyacrylamide gel (PAGE) and transferred onto an ECL nitrocellulose membrane (Amersham). The membranes were blocked overnight with phosphate saline buffer (PBS)-Tween 20 (PBST), 4% skim milk (blotto buffer) and incubated with a polyclonal mouse antiserum specific for the *gD-Ec*. Blots were then incubated with a HRP-labeled anti-mouse IgG antibody (Amersham) and developed with X-film (Kodak) using the ECL Western blotting detection system (Amersham). Concentration of the recombinant proteins was estimated from blots in a Biorad Fluor S MultiImager System using the Quantity One 4.0 software (BioRad).

### 2.3. Animal immunizations

#### 2.3.1. Mice

Male BALB/c mice (6–8 weeks old) were used for vaccination. Animals received a single dose of an oil-based vaccine formulated with incomplete Freund adjuvant (IFA) by the intra-peritoneal (i.p.) route. Fresh plant tissue was macerated in the presence of liquid nitrogen, resuspended phosphate saline buffer (PBS) pH 7.5 and emulsified in the oily phase. Mice received 0.2 ml of this vaccine containing 1 g of fresh leaf tissue/ml. A control group of animals were also vaccinated with 0.2 ml of a conventional oil BHV-1 vaccine (conv. vac.) formulated in IFA and containing inactivated BHV-1 with a previous titer of $10^{7.5}$ tissue-culture infective dose 50% ($TCID_{50}$)/ml. Animals were bled at different times post-vaccination.

#### 2.3.2. Cattle

Twelve to fourteen-month-old Holstein and Angus × Hereford cows were utilized in the immunization experiments. Animals were selected based on their negative serology for BHV-1. Cattle were immunized at days 0, 21, 45, 70, 120, 150 and 195 using 5 ml of a similar vaccine as described for mice. Half of the dose was injected by the intra-muscular (i.m.) route and the other half was injected subcutaneosly (s.c.). Positive control animals were immunized using 5 ml of the conventional BHV-1 vaccine, formulated with inactivated virus and further revaccinated 3 weeks later with the same immunogen. Serum samples were extracted from peripheral blood at different time-points after the initial inoculation.

### 2.4. Analysis of antibody response to plant-expressed gDc protein

Serum samples from mice and cattle inoculated with the plant-derived gDc were tested by Western blot and ELISA for reactivity against different BHV-1 antigens.

#### 2.4.1. Western blot

Recombinant *gD-Ec* proteins were applied to a 12.5% SDS-PAGE, blotted onto an Immobilon P (Millipore) membrane and then incubated overnight in blotto buffer at 4 °C. The next day, membranes were washed and incubated for 2 h at 37 °C with serum samples from immunized cattle diluted 1/20 in the same buffer. Blots were washed again and then incubated with an AP-labeled anti-bovine Ig guinea pig antiserum (Dakkopats) for 1 h at 37 °C. After extensive washing the reaction was developed by the addition of the NBT/BCIP substrate.

#### 2.4.2. ELISA for gD-specific antibodies

Purified recombinant *gD-Ec* diluted in 0.1 M carbonate–bicarbonate buffer pH 9.6 (0.875 μg per well) was adsorbed to Immulon II plates (Dynatech) by overnight incubation at 4 °C. Plates then were blocked with PBS-Tween 20 1% gelatin (blocking buffer) and subsequently incubated for 1 h at 37 °C with mice serum samples and then a secondary anti-mouse-Ig-specific HRP-conjugated antibody (KPL). Primary and secondary antibodies were diluted with the same blocking buffer. The reaction was developed by addition of *o*-phenylenediamine (1,2-benzenediamine) dihydrochloride-$H_2O_2$ in phosphate–citrate buffer pH 4.5 and read 3 min later at 490 nm in an MR 500 Microplate Reader (Dynatech). Titers were expressed as log 10 of the reciprocal of the highest serum dilution which gave optical density (OD) readings of at least twice a pool of sera from three animals immunized with extracts of plants inoculated with the empty vector (TMV-30B) at the same dilution.

#### 2.4.3. ELISA for BHV-1-specific antibodies

Immulon I plates (Dynatech) were incubated overnight at 4 °C with partially purified BHV-1 diluted in 0.1 M carbonate–bicarbonate buffer pH 9.6, as described before [18]. The next day, plates were blocked for 1 h with PBST 1% egg albumin, and mice or cattle serum samples were then incubated for 30 min at 37 °C. After washing, anti-mice-Ig or bovine-Ig-specific HRP-conjugated antibodies were diluted in the same blocking buffer, added to the plates and incubated for 30 min at 37 °C. The reaction was developed at room temperature by addition of 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)-$H_2O_2$ in citrate buffer pH 5 and the OD at 410 nm was measured 20 min later in an MR 500 Microplate Reader (Dynatech). Titers for mice serum samples were calculated as described for the gD-specific ELISA. Titers for bovine sera were expressed as log 10 of the reciprocal of the highest

serum dilution which gives OD readings 40% above a pool of sera (diluted 1:2560) from BHV-1 vaccinated animals.

### 2.5. BHV-1 production and purification

BHV-1 strain Los Angeles (LA) was propagated in Madin Darby bovine kidney (MDBK) cells grown in Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum (FBS; Gibco). Confluent monolayers were inoculated with BHV-1 at a multiplicity of infection of 0.1 and the virus was allowed to adsorb for 45 min at 37 °C before the addition of fresh culture medium. After a cytopathogenic effect (CPE) was observed, the supernatant was collected and cellular debris was removed by centrifugation at 3000 × *g* for 30 min. For vaccine formulation, virus was diluted to $10^{7.5}$ tissue-culture infective dose 50% and then inactivated with BEI [18]. For ELISA, supernatants were pelleted by ultracentrifugation at 120,000 × *g* for 1 h at 4 C. For in vitro lymphoproliferation assays, virus suspensions were diluted to $10^5$ $TCID_{50}$/ml, stored at −70 °C and immediately before use, inactivated by UV light exposure for 1 min at 11 cm from the source (General Electric G875 UV bulbs).

### 2.6. In vitro lymphoproliferation assay

Blood samples were collected by venipuncture with syringes containing heparin. Lymphocyte-enriched cells were isolated from the buffy coats by centrifugation on Lymphoprep™ (density 1.077 g/ml; Nycomed Pharma A.S). Cells were then washed twice in RPMI 1640 medium supplemented with 5% of foetal bovine serum and finally resuspended at a concentration of $2.5 \times 10^6$ cells/ml in the same culture medium with 10% FBS. Inactivated BHV-1 virus antigen was diluted 1:100 in RPMI 1640 medium and added to the cultures containing $2.5 \times 10^5$ lymphocytes per well, reaching a final volume of 200 μl per well. Lymphocytes were incubated for 4 days at 37 °C in a humidified atmosphere with 5% $CO_2$. Cell proliferation was measured by addition of 0.4 μCi [$^3$H]thymidine per well 20 h before harvesting. Each test was performed in triplicate. Thymidine uptake for each culture was determined in a liquid scintillation counter (LKB, Wallac, 1219 Rackbeta), and arithmetic means and standard deviations were calculated for triplicate values. Results were obtained as mean counts per minute (cpm) and expressed as stimulation index (SI = mean cpm of cultures in the presence of BHV-1 antigen/mean cpm of cultures without BHV-1 antigen). The threshold value was established at SI = 3.

### 2.7. Challenge experiments

Animals were challenged at 210 dpi using BHV-1 virus (LA strain) suspended in Eagle's medium at a concentration of $10^{7.5}$ $TCID_{50}$. A volume of 1.5 ml of the inoculum was administered into each nostril of individual animals by aerosol exposition using an ultrasonic nebulizer (Electrolab AP-300). Prior to challenge, animals were clinically examined, rectal temperature recorded and blood samples and nasal swabs were collected to establish baseline values. Nasal swabs were obtained by inserting tampons into the ventral meatus of the nasal passage and immediately dipping them in MEM containing 5000 IU penicillin/ml, 2500 μg streptomycin/ml and 10 μg amphotericin B/ml. Samples were collected 2, 4, 5, 7, 9, 11, 12, and 15 days post-challenge (dpch). For virus detection, serial dilutions of the nasal fluids were immediately inoculated onto MDBK cell monolayers cultured in 96 well plates. Monolayers were inspected for 48 h for the appearance of cytopathological effect and results were expressed as $TCID_{50}$/ml of nasal fluid. Cattle were clinically examined for 15 consecutive days after challenge and evaluated for the presence of anorexia, increase of body temperature, rhinitis and vulvovaginitis.

## 3. Results

### 3.1. Expression of recombinant gDc in plants

Infective RNA transcripts were synthesized from the TMV-gDc and TMV-30B plasmids and utilized for inoculation of *N. benthamiana*. Infected plants were isolated in growth chambers and inoculated leaves were harvested 1 week after infection. In order to analyze the production of the recombinant gDc, total proteins were extracted, separated in a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. An equivalent amount of protein extracted from 6 mg of fresh leaf tissue was loaded for all of the TMV-gDc, TMV-30B and mock infected samples. A sample with 50 ng of *gD-Ec* protein served as a positive control. Blots were analyzed by a Western assay using a mouse anti-*gD-Ec* as the probe. Results in Fig. 2 show the presence of a positive signal with similar electrophoretic

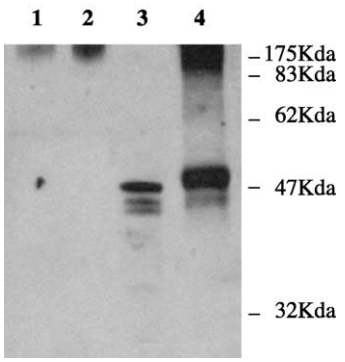


Fig. 2. Expression of the recombinant gDc protein in plants. Western blot of plant extracts and recombinant gDc developed using a mouse polyclonal serum against the *gD-Ec*. Foliar extracts from mock infected (lane 1), TMV-30B inoculated (lane 2), and TMV-gDc inoculated (lane 4) plants were blotted together with 0.05 μg of *gD-Ec* (lane 3). All plant samples derived from 6 mg of fresh leaves. Position of protein molecular weight standards is indicated on the right side of the blot.

mobility in both TMV-gDc and *gD-Ec* lanes. The amount of the recombinant gDc was estimated at about 15–20 μg/g of fresh tissue. No signal was detected for the protein extracts from TMV-30B or mock infected leaves.

### *3.2. Induction of an anti-BHV-1 response in mice immunized with crude foliar extracts inoculated with the TMV-gDc*

Mice were immunized using an oil-based vaccine containing 0.2 g of fresh leaf tissue per dose. Serum samples were recovered at different times post-immunization with the purpose of measuring reactivity against BHV-1 antigens. The recombinant *gD-Ec* was utilized as the antigen in an indirect ELISA to monitor the titre of anti-gDc antibodies induced in the mice after injection of the plant extracts. The results in Fig. 3a and b demonstrate that all animals immunized with a single dose of the plant-produced gDc developed a specific and significant humoral response to both gDc and BHV-1, presenting a kinetic that clearly resembles that of the animal immunized with a conventional vaccine for up to 80 dpi.

### *3.3. Anti-BHV-1 humoral immune responses in cattle immunized with crude foliar extracts inoculated with the TMV-gDc*

Cows serologically negative for BHV-1 were immunized with single vaccine doses consisting of a total of 5 g of fresh leaf tissue infected with either TMV-gDc or TMV-30B. Western blot analysis revealed, after two vaccinations, that the plant-derived gDc was able to induce a specific antibody response in cattle directed against recombinant *gD-Ec*. Moreover, the antibody reactivity of the plant-gDc immunized animals was similar to that of a pool of BHV-1 vaccinated cattle. As expected, cows inoculated with the control TMV-30B did not have antibodies that reacted against the BHV-1-specific bands (Fig. 4). An ELISA using whole virus as antigen was utilized to evaluate the antibody response directed against whole BHV throughout the experimental period. These results confirmed that cattle immunized with the plant-produced gDc were also able to develop a specific and significant response against the native antigen (Fig. 5). To control for possible contact of the test animals with BHV-1

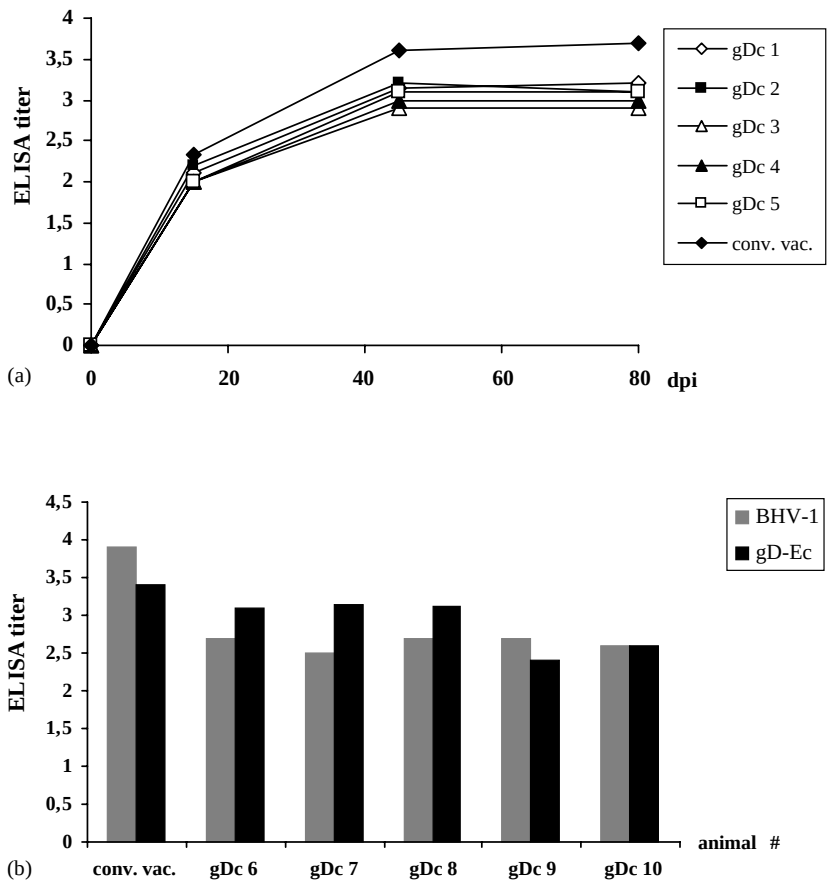


Fig. 3. Humoral immune response induced by the plant-produced gDc in mice. Titers are expressed as described in Section 2. (a) Samples were tested by ELISA for presence of anti BHV-1 gD antibodies. Animals were immunized with a conventional BHV-1 vaccine (conv. vac.) or immunogens containing fresh leaf extracts from plants inoculated with TMV-gDc (gDc1–gDc5). The titers are expressed relative to those of the control animals injected with TMV-30B, (b) Comparative response to BHV-1 whole particles and gD protein at 70 dpi. Mice were immunized as indicated above and serum samples were tested by ELISA using either partially purified BHV-1 particles (grey bars) or gD-Ec (black bars) as antigens adsorbed to the plates.

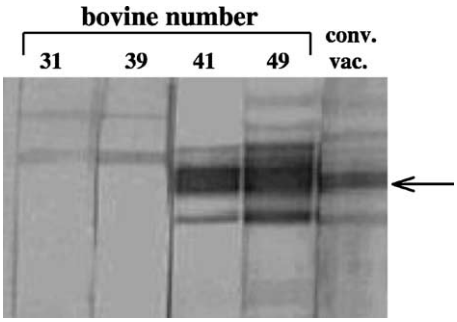


Fig. 4. Immune response (40 dpi) of cattle inoculated with the recombinant gDc produced in plants. Sera were tested by Western blot against the purified gD-Ec. Cows 31 and 39 were immunized with foliar extracts from TMV-30 infected plants and animals 41 and 49 received extracts from TMV-gDc infected plants. The positive control represents a pool of sera from five cows immunized with a conventional BHV-1 vaccine (conv. vac. #1–5). The arrow indicates the position of the *gD-Ec* protein.

in the field during the course of the experiment, a commercial ELISA system was used to test the reactivity of all the animals against the gE antigen of BHV-1 (data not shown).

### *3.4. Anti-BHV-1 cellular immune responses in cattle immunized with crude extracts from leaves inoculated with the TMV-gDc*

Lymphocyte samples from PBMC were obtained at 80 dpi from cattle injected with crude foliar extracts from TMV-gDc and TMV-30B infected plants. An additional animal inoculated with a commercial vaccine was also included in the experiment as positive control. In vitro lymphoproliferation was measured by assessing the amount of label incorporated by the cells in the presence of inactivated BHV-1 particles after 96 h of culture. Lymphocytes from animals inoculated with the plant-produced gDc exhibited positive proliferation indexes (mean SI = 3.6 and 7.0) comparable to the BHV-1 vaccinated cow (mean SI = 4.9). Cows inoculated with the TMV-30B-infected foliar extracts presented a mean SI = 1.1, under the threshold for positive responses (SI = 3.0) (Fig. 6).

### *3.5. Challenge experiments in cows vaccinated with TMV-gDc inoculated foliar extracts*

Finally, to test for the possible protective effect of the recombinant vaccines, animals vaccinated with the different foliar extracts were challenged with infective BHV-1 at 210 dpi. Protection levels obtained were assessed from the amount of excreted virus in nasal fluids (Fig. 7). Unvaccinated control animals and cows vaccinated with TMV-30B alone showed similar patterns of virus shedding. Interestingly, virus shedding was delayed up to 5 days in cattle that received plant extracts containing gDc and virus titers were reduced between 100 and 10,000 times in these animals. Moreover, two of the BHV-1 vaccinated animals (conv. vac. #2 and #4) showed similar and even higher virus excretion titers than the plant-gDc inoculated cows. These results were affirmed with the observation that clinical symptoms appeared later and were milder in the plant-gDc vaccinated animals (data not shown).

## 4. Discussion

We have demonstrated here that a plant viral based vector can be used to produce sufficient quantities of an animal viral antigen in crude plant extracts to be useful for effectively immunizing animals against animal viral pathogens.

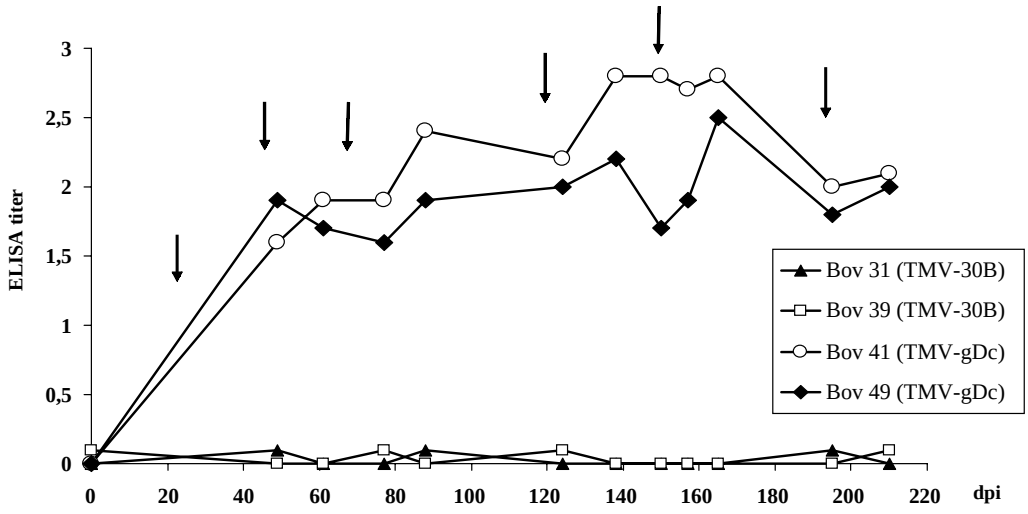


Fig. 5. Humoral immune response induced in cattle injected with plant extracts. (a) Serum samples were extracted at different time-points and tested by ELISA using whole BHV-1 particles as antigen. Primary vaccination and subsequent booster injections (indicated by arrows) were performed using 5 g of fresh leaf tissue from plants inoculated with TMV-gDc (animals 41 and 49) or TMV-30B (animals 31 and 39).

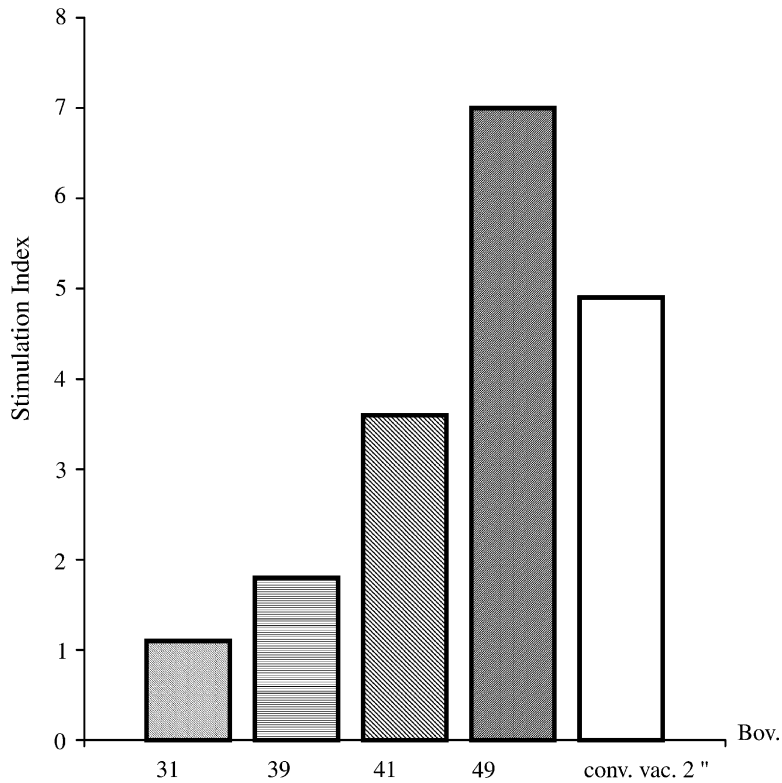


Fig. 6. Cellular immune response induced in cattle to the plant-produced gDc. Lymphocyte-enriched cell cultures were prepared from PBMC at 80 dpi. Blood cell samples were extracted from cattle immunized with crude foliar extracts from plants infected with TMV-gDc (bovines 41 and 49), TMV-30B (animals 31 and 39), or from one animal injected with the BHV-1 conventional vaccine (conv. vac. #2). For each animal, triplicate cell cultures were incubated with or without the specific BHV-1 antigen. Stimulation indexes in each case were determined as a ratio between the arithmetic means of cultures with and without antigen (SI = 3 are considered positive).

Previously, we demonstrated that protective immunity could be produced in mice injected with plant extracts containing VP1 of foot-and-mouth disease virus expressed from the same TMV vector [15]. Together, these two examples, using two very different proteins derived from unrelated RNA and DNA virus candidates, provide good evidence that animal viral proteins expressed in plants retain sufficient structural integrity to be useful for eliciting immunologically useful responses in test animals.

First, our results showed quite convincingly that the TMV vector carrying a cytoplasmic form of the glycoprotein D subunit of bovine herpesvirus was able to direct the synthesis of a protein of the expected electrophoretic mobility (ca. 55 kDa) and antigenic identity at concentrations estimated in the range of 20 μg/g of fresh tissue. The protein appeared to be stable in the plant extracts and slightly larger in size than the *E. coli*-expressed gD. We assessed if the recombinant plant-produced gDc retained a proper conformation by testing its antigenic properties by performing a series of immunological experiments in both an animal model and the natural host.

The antigenicity of the recombinant gDc was initially studied in a murine model. A group of 10 animals received a single dose of a vaccine formulated with crude extracts containing approximately 2 μg of the plant-gDc. All the animals developed a specific and sustained immune response against both the gDc and the BHV-1, for up to 80 dpi. This response was comparable to that registered for mice vaccinated with the conventional immunogen, indicating that the plant-gDc was immunogenic in mice and retained B-cell epitopes that were also present in the native virus particle. Analysis of the BHV-1-specific IgG isotype also demonstrated the presence of predominant levels of both IgG1 and IgG2a and to a lesser extent IgG2b and IgG3 (data not shown). In contrast, animals immunized with the inactivated-virus vaccine presented only significant levels of IgG1. These results indicate the presence and functionality of T-cell epitopes in this plant-produced gDc immunogen. This is consistent with the high and persistent level of specific antibodies induced by this protein.

Based on the positive results in mice, we then examined the immune response induced by the plant-gDc in the natural bovine host. Cattle were immunized using the same crude immunogen, but they received a larger dose and a greater number of booster doses because of the large size of the animals. Both of the cows immunized with the gDc containing plant extracts developed significant ELISA titers against the recombinant *gD-Ec* by 40 dpi. This anti-gDc antibody response strongly correlated with the specific anti BHV-1 humoral response measured at 50 dpi and the positive

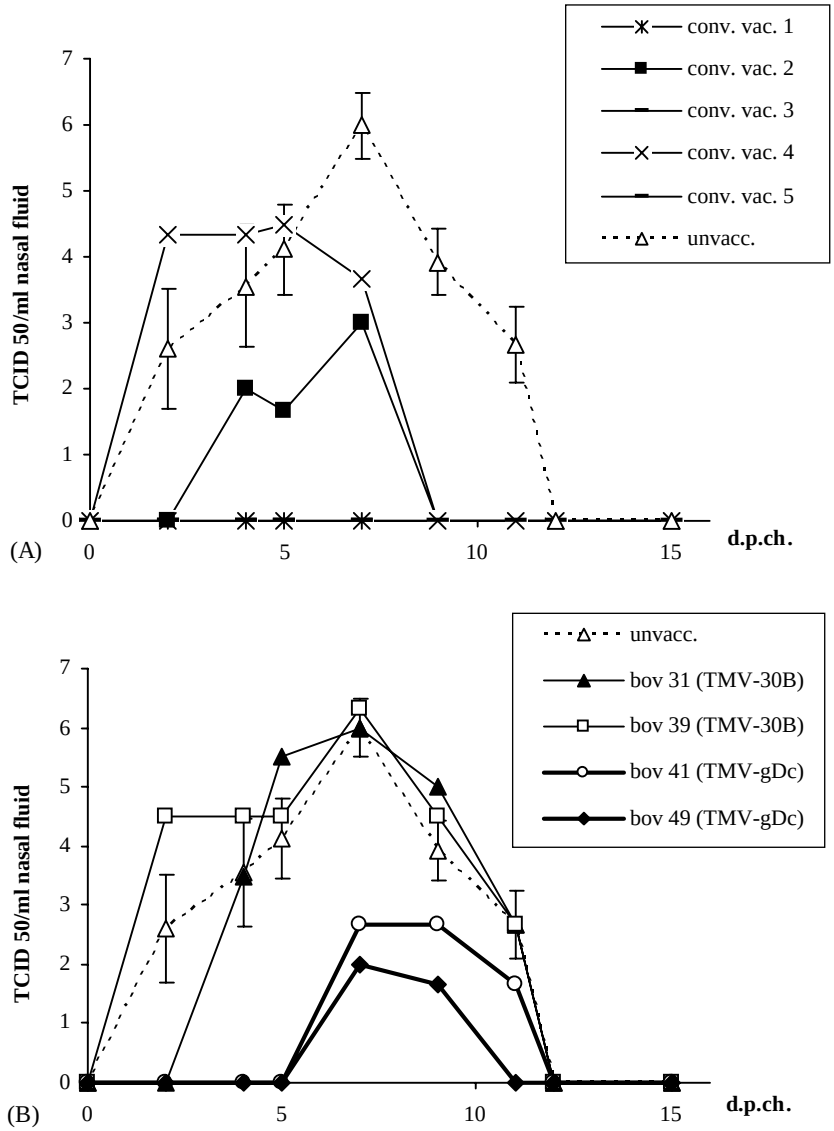


Fig. 7. Virus excretion in cows after challenge with infective BHV-1. Cattle were challenged at 210 dpi and the presence of infective virus in nasal excretions was assessed for each animal. The titers obtained are presented separately for each animal, except for unvaccinated controls. Both panels: the unvaccinated control results represent the mean titer (±S.D.) obtained from four animals. Panel A: animals vaccinated with the BHV-1 conventional vaccines (animals 1–5) or with extracts from plants. Panel B: animals vaccinated with extracts from plants inoculated with TMV-30B (bovines 31 and 39) or TMV-gDc (bovines 41 and 49).

cellular immune response evaluated at 80 dpi. Moreover, cattle immunized with the plant-produced gDc presented good levels of protection to viral challenge. These results were consistent with the observed cellular response, as it is generally established that stimulation of the cellular immunity plays a central role in the protection against the BHV-1 infection [27]. Both the emergence of symptoms and detection of virus in nasal excretions were significantly delayed and shortened in these animals compared with controls. Importantly, gDc-vaccinated cattle presented much less severe symptoms and virus shedding in nasal fluids was reduced by 2–4 logs in comparison with negative controls. Conventionally vaccinated animals showed variable levels of protection, as previously reported [26]. While three of them did not show significant virus shedding, the other two showed an early detectable virus excretion. A most interesting result was that protection levels reached in cows immunized with the plant-derived antigens—in terms of length, delay and reduction of virus excretion—were similar to or even better than some of animals that received the conventional vaccine. Our results contrast with previous reports which were unsuccessful at eliciting a level of protective response as presented here when they used an unglycosylated form of the BHV gD produced in *E. coli* [22].

This is the first report showing induction of protective antibodies in cattle immunized with a plant-produced BHV-1

antigen. In conclusion, we have now demonstrated that the TMV-30B vector is suitable for production of proteins with moderately high molecular weight such as gDc. The immunological characterization of the protein showed that a significant part of the native gD conformation was retained in the recombinant plant protein. These results, along with those reported previously for FMDV demonstrate that a structural protein of an RNA virus and an external membrane protein of a DNA virus can be expressed in plants in sufficient quantity and of sufficient quality to be useful for eliciting protective antibodies in vaccinated animals. We recognize that that this relatively simple technology needs to be further refined in order to be put into practice for production of low cost and biologically safe animal vaccines. Although optimization of the technology for commercial production of useful vaccines utilizing this technology is beyond the scope of this study, a recent report suggests that this goal could be effectively accomplished through a commercial partnership (personal communication, Greg Pogue, Large Scale Biology Corporation). In this era of concern over the deliberate release of animal pathogens for the purposes of bioterrorism, it would seem prudent for regulatory agencies to further explore this relatively inexpensive approach for producing agriculturally important vaccines.

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