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Research paper

Efficacy of a BVDV subunit vaccine produced in alfalfa transgenic plants

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is considered an important cause of economic loss within bovine herds worldwide. In Argentina, only the use of inactivated vaccines is allowed, however, the efficacy of inactivated BVDV vaccines is variable due to its low immunogenicity. The use of recombinant subunit vaccines has been proposed as an alternative to overcome this difficulty. Different studies on protection against BVDV infection have focused the E2 protein, supporting its putative use in subunit vaccines. Utilization of transgenic plants expressing recombinant antigens for the formulation of experimental vaccines represents an innovative and cost effective alternative to the classical fermentation systems.

The aim of this work was to develop transgenic alfalfa plants (*Medicago sativa*, L.) expressing a truncated version of the structural protein E2 from BVDV fused to a molecule named APCH, that target to antigen presenting cells (APCH-tE2). The concentration of recombinant APCH-tE2 in alfalfa leaves was 1 μg/g at fresh weight and its expression remained stable after vegetative propagation. A methodology based an aqueous two phases system was standardized for concentration and partial purification of APCH-tE2 from alfalfa. Guinea pigs parentally immunized with leaf extracts developed high titers of neutralizing antibodies. In bovine, the APCH-tE2 subunit vaccine was able to induce BVDV-specific neutralizing antibodies. After challenge, bovines inoculated with 3 μg of APCH-tE2 produced in alfalfa transgenic plants showed complete virological protection.

1. Introduction

Bovine viral diarrhea (BVD) infection, caused by a Pestivirus of the Flaviviridae family, is an important cause of morbidity and economical losses in cattle worldwide. Bovine viral diarrhea virus (BVDV) infections are associated with several consequences, such as fertility problems, immunosuppression, diarrhea, thrombocytopenia, and, frequently, unapparent courses (Baker, 1995; Thiel et al., 1996). Transplacental infection can lead to abortion, stillbirth, malformation or persistent infection of the calves.

Performance of commercially available inactivated vaccines is a controversial issue due to a variety of reasons

Abbreviations: BVDV, bovine viral diarrhea virus; APCH-E2t, a truncated version of protein E2 fused to APCH molecule; NAb, neutralizing antibodies; MDBK, Madin–Darby bovine kidney; PMSF, phenylmethylsulfonyl fluoride; ATPS, aqueous two-phase partitioning system; CsMV, cassava striate mosaic virus; TSP, total soluble protein.

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such us the absence of experimental evidence of protection levels during different outcomes of the infection and the diversity of circulating strains (Bolin, 1995) and a recent report of the association of an inactive vaccine with an emerging disease named bovine neonatal pancytopenia (Deutskens et al., 2011). Therefore, the introduction of more efficacious vaccines is demanded by veterinarians and farmers. Subunit vaccines provide an opportunity to develop safe and rational vaccines, with the possibility of differentiating between vaccinated and infected animals. However, the challenge is to produce a vaccine capable of eliciting an efficient immune response at an affordable cost for veterinary applications.

E2 is the major glycoprotein of BVDV envelope and the most immunogenic protein of the virus. This glycoprotein contains the main antigenic sites for the production of neutralizing antibodies (NAb) which play a preponderant role in the defense against infection (Deregt et al., 1998; Fulton et al., 1997; Paton et al., 1992). Immunization with E2 induces NAb in experimental and natural hosts. Moreover, it has been demonstrated that NAb raised against E2 are able to protect susceptible hosts from infection with BVDV (Ferrer et al., 2007; Kweon et al., 1997; Rosas et al., 2007; Thomas et al., 2009).

Thus, E2 glycoprotein constitutes an excellent candidate for the development of an experimental subunit vaccine. Unfortunately, none of the eukaryotic expression systems currently available allows the scale-up production of recombinant glycoprotein at a cost effective for veterinary industries.

The use of transgenic plants for the production of viral and bacterial antigens has been frequently reported during the last 10 years (Chia et al., 2010; Daniell et al., 2009; Floss et al., 2007; Rybicki, 2009; Tiwari et al., 2009). This biotechnological strategy has the enormous advantage of not requiring fermentation systems under strict conditions of biosafety and sterility, which makes this technology specially attractive for its simplicity and low cost. Moreover, plants are capable of performing post-translational modifications including glycosylation required for recombinant protein bioactivity (Gomord et al., 2010; Karnoup et al., 2005).

In this work, a truncated version of BVDV glycoprotein E2 fused to a molecule that target to antigen presenting cells (APCH-tE2) (Gil et al., 2011; Ostachuk et al., 2009) was expressed in transgenic alfalfa plants. The recovery and concentration of the antigen from leaf extracts was performed using an aqueous two phases partition system. Immunogenicity of APCH-tE2 vaccine was evaluated in guinea pigs and the natural host. Immunized bovines were challenged with BVDV and protection against infection was assessed.

2. Materials and methods

2.1. Virus

The NADL strain of BVDV-1 (ATCC) and an Argentinean non-cytopathic isolate (98/124) were cultured in Madin–Darby bovine kidney (MDBK) cells. The culture medium consisted of E-MEM supplemented with 100 U/ml penicillin, 100 (g/ml streptomycin and 2% heat inactivated FBS (Internegocios S.A.). Virus stocks were clarified by centrifugation at 600 × *g* and stored at −80 °C.

2.2. Production of transgenic plants of alfalfa containing the BVDV-E2t gene

Total RNA from BVDV (NADL strain) infected MDBK cells was isolated and a reverse transcription was performed using hexa random primers (Biodynamics SRL, Argentina). The primers used for the PCR were synthesized according to the coding sequence of BVDV-1 NADL strain obtained from the NCBI (ACCESSION: NC_001461). The tE2 sequence was amplified with a *Bam*HI restriction site at 5′ end (sense primer, 5′-CGC GGA TCC GTA CAG GGC ATT CTG-3′) and a *Xho*I restriction site at 3′ end (antisense, primer 5′-GTA CTC GAG CTC AGC GAA GTA ATC-3′).

The tE2 coding sequence was cloned in the MCS of a modified version of pBI121 vector (Clontech) (pCs) in *Bam*HI/*Xho*I restriction sites (Fig. 1A). Briefly, uadA gene from pBI121 was replaced by a multiple cloning site containing *Bam*HI, *Sma*I, *Stu*I, *Spe*I, *Sca*I and *Xho*I sites. In addition, the CaMV (Cauliflower mosaic virus) 35S promoter was replaced by the CsMV (Cassava striate mosaic virus) promoter. This promoter was obtained from plasmid pILTAB381, kindly provided by Samac et al. (2004). APCH sequence was obtained from the vector pGEM-APCH1 (Gil et al., 2011) and cloned upstream the tE2 sequence.

The obtained recombinant binary vector, pCs–APCH-tE2, was introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation, using the procedure previously described (Wigdorovitz et al., 1999). Petioles of alfalfa clone C23, were co-cultivated with *A. tumefaciens* and cultured *in vitro* as described by McKersie et al. (1993). The *in vitro* selection was performed using 25 mg/l of kanamycin

2.3. Genetic analysis of the recombinant plants

The presence of the recombinant gene in the kanamycin resistant plants was detected by PCR. Total nucleic acids were extracted from samples of approximately 50 mg of leaves following a protocol previously described (Wigdorovitz et al., 1999). The following pair of primers: forward primer 5′ CGCGGATCCGTACAGGGCATTCTG 3′; reverse primer 5′ CCCATGGGGTAGTGTGG 3′ were used to specifically amplify a 300 bp DNA fragment of the E2 gene. The presence of the *npt*II gene, which confers resistance to kanamycin, was also evaluated by PCR (Wigdorovitz et al., 1999).

The transcription of the transgenes was analyzed by RT-PCR. Total RNA extraction was performed from 1 g of fresh leaves following the protocol described elsewhere (Wigdorovitz et al., 1999). PCR was performed using the same primers described above for detecting the presence of E2 gene in the plants. As positive control for RNA extraction, a fragment of approximately 800 bp which encompasses the 5.8S mRNA gene and the flanking intragenic transcribed spacers was amplified using specific primers (forward primer: 5′ GGAAGGAGAAGTCGTAACAAGG 3′, reverse primers: 5′ TCCTCCGCTTATTGAT ATGC 3′). The

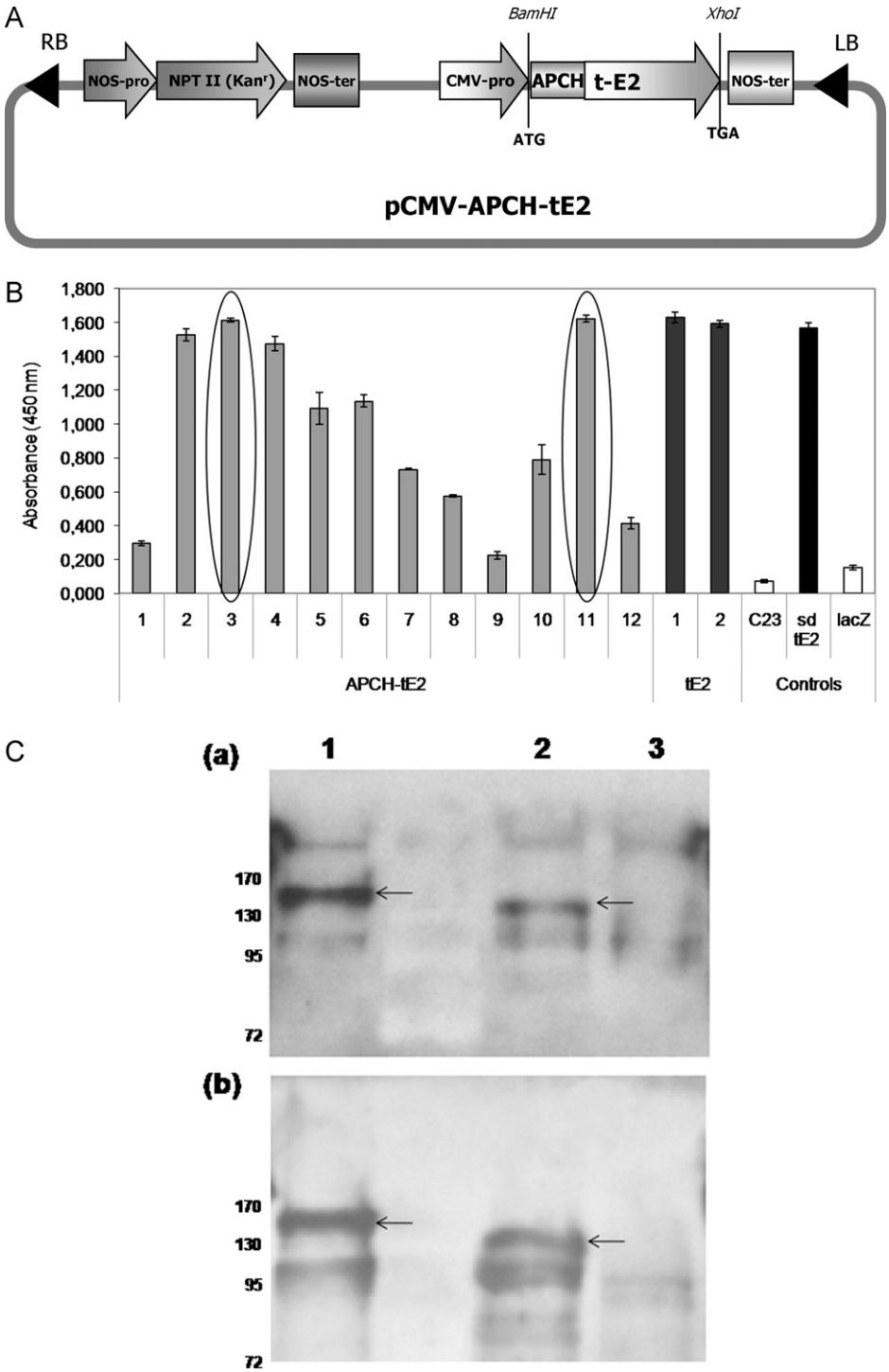

Fig. 1. Generation of transgenic alfalfa plants expressing APCH1-tE2. (A) Schematic representation of the pCs–APCH1-tE2 plasmid. (B) Detection of the recombinant protein APCH-tE2 by ELISA. Leaf extracts from independent transgenic events were tested for their reactivity in ELISA as described in the text. Each bar in the histogram represents an individual transgenic plant (which is identified by the number at the bottom of the bar). As an example, the analysis of 12 plants transformed with APCH-tE2 is plotted. tE2 1 and 2: tE2 expressed in transgenic alfalfa without the APCH-molecule fused. Controls: nt: non-transformed alfalfa plants. sdtE2: tE2 from stably transformed CHO-K1 cells. lacZ: supernatant of CHO-K1 cells stably transformed with lacZ gene. (C) Detection of the recombinant APCH-tE2 expression in selected transgenic plants by Western blot analysis. Reactivity from leaf extracts from selected plants (Nos. 3 and 11) was assessed with mAb C2.9.H (a) and by polyclonal rabbit antiserum against tE2 protein (b). Lane 1: APCH-tE2 expressed in CHOK1 cell line, lane 2: APCH-tE2 from transgenic plants, lane 3: supernate from a negative plant. The position of the molecular weight marker (Bio-Rad Broad Range) is indicated at the left. Arrows indicate the position of APCH-tE2 dimers from plant extracts and APCH-tE2 dimers from mammal cells.

same nptII primers described above for the PCR technique were used as an additional control of RNA detection.

2.4. ELISA for APCH-tE2 screening and quantitation

Plant extracts were prepared by grinding 100 mg of freshly harvested plant tissue in the presence of liquid nitrogen and the resulting powdered leaves were suspended in 300 μl extraction buffer (PBS, 5 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1% sodium Ascorbate, 0.2% Tritón X-100) at 4 °C. The extracts were clarified by centrifugation at 5000 rpm for 10 min at 4 °C. The extracts were assayed in an indirect sandwich-type ELISA previously developed (Pecora et al., 2009). Statistical significance was assessed using Student *t*-test at $p < 0.05$ for all comparisons, using the Statistix 8.0 Analytical Software.

2.5. Antigenic analysis of the recombinant plants

The expression of the APCH-tE2 protein in the transgenic plants was analyzed by Western blot in non-reducing and denaturing conditions. Plant extracts were prepared as described above, and suspended in SDS-PAGE sample buffer (50 mM Tris, pH 7.5, 1 mM PMSF, 8 M urea, 1% SDS), boiled 5 min, electrophoresed in 10% SDS-PAGE and blotted onto an Immobilon P (Millipore) membrane. The membrane was blocked overnight with PBS containing 0.05% Tween-20 (PBS-T), 3% skimed milk (all subsequent steps were performed using this buffer) and incubated with the monoclonal antibody (mAb) H2.9 against the E2 glycoprotein (Marzocca et al., 2007) and a rabbit anti-tE2 serum 1 h at 37 °C. The membrane was then washed using PBS-T, and incubated with a peroxidase labeled anti-mouse Ig goat antiserum (KPL) and peroxidase labeled anti-rabbit Ig goat antiserum (KPL), respectively for 1 h at 37 °C. After washing four times, the reaction was developed by the addition of the ECL substrate (PerkinElmer).

2.6. Vegetative propagation

The selected plants were propagated in order to increase the leaf biomass.

Five to 10 cm lengths of alfalfa stems which contains two or three petioles were cut for rooting. The end of the cutting was dipped into ddH_2O and then into a 0.1% auxin rooting hormone. After that, the stem cutting was placed into sterile soil saturated with water and the pot was placed into a mist chamber in the greenhouse.

When the cuttings have rooted they were removed from the mist chamber and were transplanted to a larger pot and placed in the greenhouse.

2.7. Antigen preparation

The aqueous two-phase partitioning system (ATPS) was used to concentrate and purify APCH-tE2 from the plant extract. Working conditions were standardized using tE2 produced in CHO-K1 cells, purified by immobilized metal affinity chromatography. Stock solutions of PEG 4000 50% (w/w) phosphate buffer pH7 were prepared.

Twelve milliliters of plant extract were mixed with 3.9 ml PEG 4000 solution and 11.1 ml phosphate buffer. The mixture was centrifuged at 2000 × *g* during 25 min. The interphase was recovered using a peristaltic pump.

Antigen concentration was determined by an ELISA previously described (Pecora et al., 2009).

2.8. Vaccine formulation

Vaccines were formulated with oil adjuvant (Marcol Arlacel) containing the appropriate antigen in a proportion adjuvant:antigen of 60:40. As positive control, an inactivated-BVDV vaccine containing 10^7 TDIC 50 ml^{-1} BVDV-1 was included. As a negative control, the same formulation was made using non-transgenic plant extracts as antigens.

2.9. Immunization of guinea pigs

Female and male guinea pigs, 8–12 weeks old, were obtained from the animal care facilities of the CICVyA, INTA. Animals were checked for the absence of BVDV-specific antibodies by seroneutralization.

Groups of guinea pigs ($n = 5$) were immunized with (i) 0.2 μg alfalfa APCH-tE2; (ii) 0.04 μg alfalfa APCH-tE2; (iii) 0.2 μg of tE2 produced in CHO-K1 cells as positive control group; and (iv) alfalfa extract from a non-transgenic plant (n.t.) containing similar total protein mass as negative control group. Guinea pigs were immunized with 2 doses of 0.6 ml each, at days 0 and 21. Immunogens were administered by the intramuscular route (i.m.). Sera was sampled on days 0, 30 and 45. Guinea pig handling, inoculation, and sample collection were done by trained personnel under the supervision of a veterinarian and in accordance to protocols approved by the INTA's Ethical Committee of Animal Welfare (CICUAE).

2.10. Immunization of cattle

Female and male Aberdeen Angus bovines (6–8 months old) located at the Balcarce Experimental station, INTA, were checked for the absence of BVDV-specific antibodies by the seroneutralization test and BVDV antigen by RT-PCR. Vaccines were formulated as described for guinea pigs assays but using two different doses of APCH-tE2 (3 and 1.5 μg per animal). The assay included a positive control group that was immunized with a vaccine containing 10^7 TDIC 50 ml^{-1} of BVDV-1 and a negative control group which was vaccinated with a formulation of an extract from a non-transgenic plant. Groups of 3 bovines were immunized with 3 ml of each vaccine at days 0 and 30 by i.m. Sera from all animals were weekly collected until 60 days post inoculation (dpi).

2.11. Seroneutralization

Serum neutralizing antibodies were detected by virus neutralization assay. Briefly, 100 $TCID_{50}$ of BVDV (NADL strain) were co-incubated with 75 μl of 1/4 serial serum dilutions of the inactivated serum samples for 1 h at 37 °C. Then, the mixture was added to plates with 3×10^4 MDBK

cells/well. The plates were incubated for 72 h at 37 °C, 5% CO_2. Control wells without the virus were used for each serum sample in order to discard toxicity. Serum neutralization titers were determined by the method of Reed and Muench (Stanic, 1963). A BVDV positive serum from experimentally infected cattle was used as positive control, and a BVDV negative serum from a reference animal was used as negative control.

Differences in bovine antibody titers among groups were evaluated by ANOVA under a model of repeated measures throughout time, followed by a general contrast post-ANOVA test. Statistical significance was assessed at $p < 0.05$ for all comparisons, using the Statistix 8.0 Analytical Software.

Cattle management, inoculation, and sample collection were conducted by trained personnel under the supervision of a veterinarian and in accordance with protocols approved by the INTA's Ethical Committee of Animal Welfare (CICUAE).

2.12. Challenge

Calves were housed in strict isolation at an animal research facility and fed standard concentrate diet. The virus inoculum was administered 60 days post-vaccination, by inhalation, using a nebulizer to administer 25 ml of tissue culture fluid containing 10^9 $TCID_{50}$ of BVDV isolate 98/124 (type IB). For all time references this point was defined as day 0. Clinical parameters recorded included rectal temperature, alertness, appetite, respiratory rate, body condition, and presence and characteristics of diarrhea. Samples of blood, nasal and ocular swabs were taken at 0, 4, 6, 8 and 11 days post challenge. The blood was centrifuged at 125 × *g* at 4 °C for 20 min; the buffy coat and the plasma were conserved at −80 °C for further analysis.

Viral isolation assays were performed on samples from plasma, buffy coat and ocular and nasal swabs (Odeon et al., 2009). After four passages in MDBK cells, viral antigens were detected by immunofluorescence using an anti-BVDV antibody conjugated with FITC (VMRD). Results obtained by cell culture isolation were confirmed by RT-PCR, using IA and IB oligonucleotides (IA: 5′ GAGGCTAG CCATGCCCTTAGT 3′, IB: 5′ TCAACTCCA TGTGCCATGTACAGCA 3′), which amplify the fragment comprised between positions 98 and 402 of the non-coding 5′ region reported for the NADL sequence (Pellerin et al., 1994).

3. Results

3.1. APCH-tE2 expression in transgenic plants

Transgenic plants containing the APCH-tE2 gene under the CsMV promoter were produced. After 10 independently transformation experiments using *A. tumefaciens* transformed with pCMV-APCH-tE2 (Fig. 1A), 80 transgenic plants with the ability of growing in the presence kanamycin, were obtained. The expression and transcription of the APCH-tE2 gene in the transgenic plants were confirmed by PCR and RT-PCR, respectively (data not shown).

The presence of the recombinant protein in the plants harboring the foreign gene was screened by ELISA using an anti-E2 polyclonal serum. Two plants showed the highest level of APCH-tE2 accumulation (Fig. 1B) these were transgenic plants 3 and 11. The average yield of APCH-tE2 was approximately 1 μg/g of fresh biomass. The expression of APCH-tE2 protein was further analyzed by SDS-PAGE and Western blot, showing that APCH-tE2 was specifically recognized by both mAb Cevan H2.9 and rabbit anti-serum against tE2 (Fig. 1C).

3.2. Stability of expression

One hundred and twenty transgenic clones were obtained by vegetative propagation of selected transgenic plants number 3 and 11.

The expression of APCH-tE2 protein was quantified in all the plants generated, in order to assess the stability of the expression among the plants obtained by vegetative propagation. Results showed that APCH-tE2 was expressed stably and the accumulation of the antigen was similar in all the clones evaluated (Fig. 2A). No statistical differences were apparent between the clones. The expression level of the harvested selected plant remained practically unchanged when monitored during 12 month experimental period (Fig. 2B). No statistical difference was apparent over time.

After assessing the expression levels homogeneity, the culture conditions in greenhouses were established to fix biomass harvesting cycles of 21 days, reaching a performance of 1–1.3 kg/m^2 of production area.

3.3. Concentration of the antigen

With the purpose of obtaining the optimal concentration of the antigen for vaccine formulation, an aqueous two-phase partitioning system (ATPS) was standardized to obtain APCH-tE2 protein from alfalfa leaf extracts. APCH-tE2 was mostly detected in the interface, where its concentration was 10-fold higher than in leaf extract.

APCH-tE2 protein could be therefore concentrated with a final yield of 3.45 μg/ml which permitted the formulation of an effective vaccine dose for cattle immunization. Accumulation of APCH-tE2 in the interface allowed to partially purify the antigen since several plant proteins were excluded and remained in the phosphate phase (Table 1).

3.4. Guinea pig immunization

Guinea pigs were immunized at days 0 and 21 with plant extracts containing 0.2 μg and 0.04 μg of tE2-APCH protein.

Following primary vaccination, the animals produced NAb against BVDV, and after the second dose the NAb level reached titers higher than 2.4 in both experimental doses evaluated (Fig. 3). The negative control group, inoculated with an extract of non-transgenic plant, did not produce NAb against BVDV.

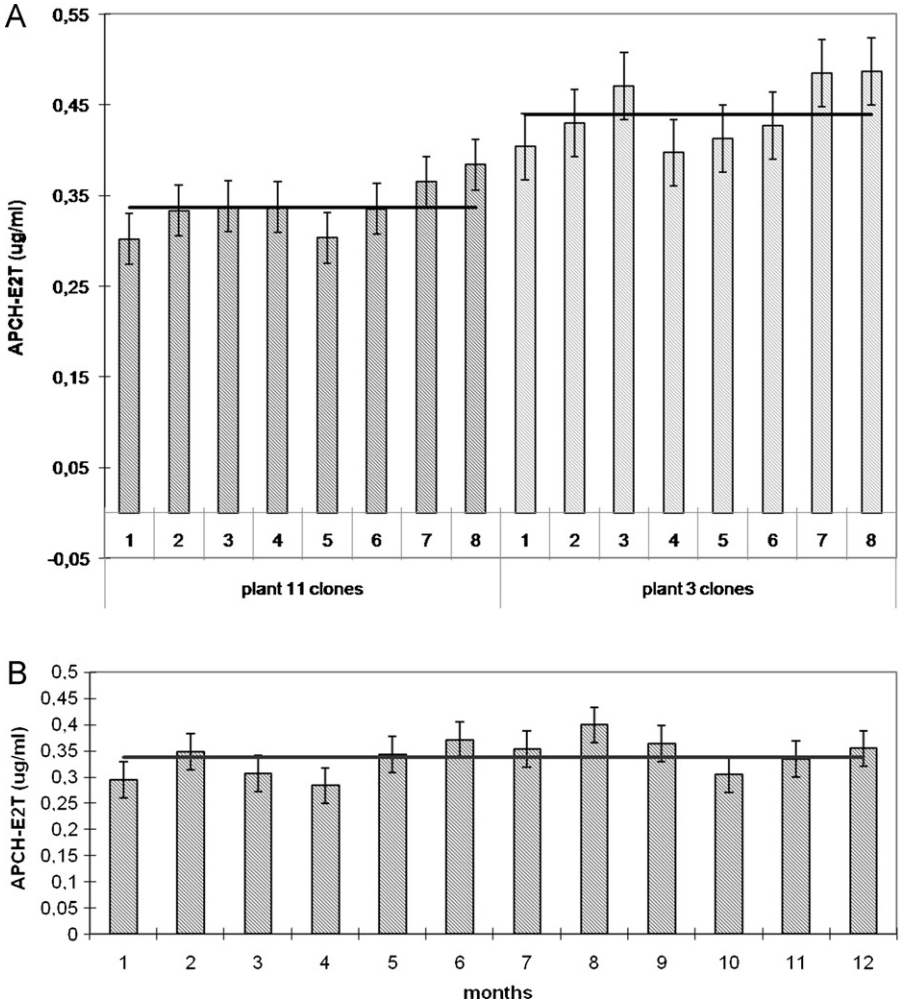

Fig. 2. Stability of expression of APCH-tE2 in alfalfa plants. (A) Stability of expression in propagated material. The expression of APCH-tE2 protein was quantified in all the plants generated in order to assess the stability of the expression among the plants obtained by vegetative propagation. Each bar represents the concentration of APCH-tE2 protein in each clone propagated. As an example, the analysis of 8 clones from each transformation events selected are plotted (repetitions $n = 3$). (B) Stability of expression over time. The level of recovery of APCH-tE2 protein from transgenic alfalfa plants was assessed over 12 months. Each bar represents the concentration of specific protein in the extraction each month and the solid line represents the average of APCH-tE2 concentration in the supernatant over time (repetitions $n = 3$). Quantification was performed through ELISA previously described.

3.5. Induction of immune response in cattle

Based on the results obtained in the experimental model, the efficacy of the APCH-tE2 subunit vaccine was evaluated in BVDV's natural host.

At 30 days after primary vaccination, early signs of a specific NAb response were observed in the groups immunized with inactivated BVDV and the higher dose of APCH-tE2. Following the second vaccination, NAb titer increased to high levels in both groups of APCH-tE2 immunized calves.

Table 1
Partial purification.

Fraction	TSP (μg/ml)	APCH-tE2 (μg/ml)	Purity	Yield (%)
APCH-tE2[a]	18,769 ± 0.91	0.54	1	100
APCH-tE2[b]	4490 ± 0.69	0.45	3.54	83.33
Interphase[c]	25,485 ± 0.59	3.45	5.88	76.67

Each fraction was evaluated by ELISA (previously described) and Bradford quick assay (Bio-Rad).
Purity: it was expressed as the ratio [APCH-tE2/TSP] relativized to APCH-tE2 (preclarification supernatant).
[a] APCH-tE2: preclarification supernatant.
[b] APCH-tE2: postclarification, interphase.
[c] Interphase from the aqueous two phases system.

Table 2
Virus isolation following BVDV challenge.

Group		BVDV			Control			APCH-tE2 (3 μg)			APCH-tE2 (1.5 μg)		
Cattle		460	461	462	446	463	467	464	482	474	476	450	466
0 dpi	BC	–	–	–	–	–	–	–	–	–	–	–	–
4 dpi	BC	–	–	–	+	+	+	–	–	–	–	+	–
	NS	–	–	–	–	–	–	–	–	–	–	–	–
6 dpi	BC	–	–	–	+	–	+	–	–	–	–	+	–
	NS	–	–	–	–	–	–	–	–	–	–	–	–
8 dpi	BC	–	–	–	–	–	–	–	–	–	–	–	–
	NS	–	–	–	+	–	+	–	–	–	–	–	–
11 dpi	BC	–	–	–	–	–	–	–	–	–	–	–	–
	NS	–	+	–	–	–	–	–	–	–	–	–	–

Following vaccination and challenge, samples were taken every 2 days for 10 days. Virus isolation from buffy coats (BC) and nasal swabs (NS) was carried out by immunofluorescence assay after four passages on MBDK cells (dpi: days post-infection).

At the time of challenge (t60), vaccinated animals presented specific NAb with titers higher than 2 (Fig. 4). In contrast, no significant neutralizing antibodies were detected in the control group and remained so until 15 days post-challenge.

Following vaccination, none of the animals showed local reactions or adverse effects.

After challenge, NAb titers remained high in the vaccinated groups, while the negative control group showed seroconvertion. At the end of the experience, 30 days post challenge, all animals displayed high NAb titers.

Total leukocyte count fell slightly in all groups between days 1 and 3 post challenge and recovered almost pre-challenge values by day 14. No statistical differences were apparent between the groups over time (data not shown).

There was some evidence for a hyperthermia in all groups, particularly in the negative control group and in the animals vaccinated with 1.5 μg of APCH-tE2. Values of rectal temperature did not differ significantly between the groups, however, in the control group one animal presented values over 40 °C for more than 2 days (data not shown).

3.6. Virus isolation

In the control group BVDV was detected in all animals on day 4 post-challenge and it was further detected on days 6 and 8 either in buffy coat or nasal swabs. In contrast, BVDV was only detected in 1 calf vaccinated with the lowest doses of APCH-tE2 and 1 animal immunized with the BVDV vaccine (Table 2). Calves immunized with 3 μg of APCH-tE2 produced in alfalfa showed complete protection against the challenge.

4. Discussion

In veterinary medicine, vaccinology addresses a wide spectrum of challenges. These include the development of cost effective strategies to prevent and control infectious diseases, taking into account animal welfare and focusing on decreasing production costs of animals used for food (Shams, 2005). The production of vaccines for veterinary use also needs low cost systems combined with inexpensive application strategies.

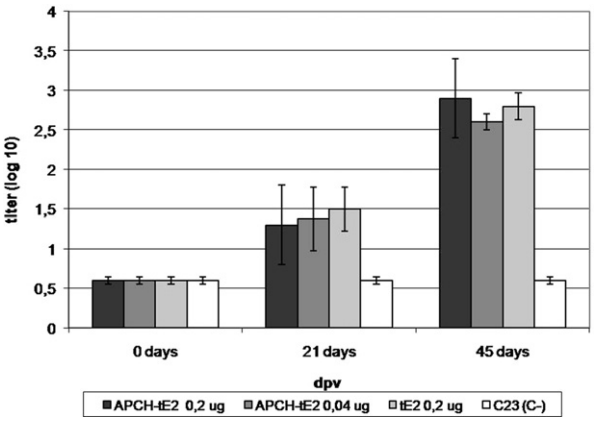

Fig. 3. Humoral immune response in guinea pigs. Experimental groups ($n = 5$) were immunized with oleous vaccines containing 0.2 μg or 0.04 μg of APCH1-tE2 a positive control group was immunized with a vaccine containing 0.2 μg tE2 produced in stable mammalian cell line and a negative control group was immunized with an extract of a non-transgenic plant (nt). At 30 and 45 days post vaccination (dpv) animals were bled and sera were evaluated by seroneutralization assay. Each bar represents the average titer of the group.

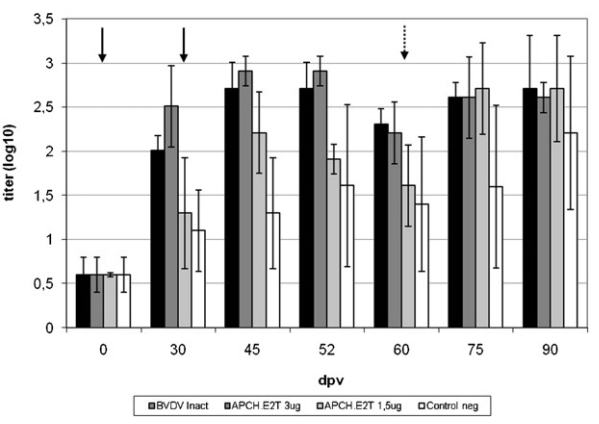

Fig. 4. Humoral immune response in cattle. Calves were immunized with 3 μg or 1.5 μg of APCH-tE2 and at 30 and 60 days post vaccination (dpv) were bled and sera were evaluated by seroneutralization assay. A positive control group was immunized with a vaccine containing 10^7 TDIC 50 ml^{-1} BVDV, NADL strain and a negative control group was vaccinated with a formulation of an extract of a non-transgenic plant (nt). Solid arrows indicate times of vaccination and the dotted arrow indicates the day of challenge. Each bar represents the average titer of the group.

Inactivated and modified-live vaccines (MLV) are used to protect cattle from BVDV infection; however, both types of vaccines have significant shortcomings. Thus, there is a need for developing more efficacious and safer vaccines. A subunit vaccine is safe in terms of horizontal and vertical transmission, and allows discrimination between vaccinated and infected animals. In this regard, over the past decade, plants have been assayed as recombinant protein production systems. Potentially, they have great advantages over microorganisms, and animal cell systems, in terms of production scale and economy, product safety and ease of storage and distribution (Ashraf et al., 2005; Daniell et al., 2001). Moreover, unlike bacteria, they are capable of eukaryotic post-translational modifications, most importantly glycosylation (Karnoup et al., 2005)

Since first described by Mason et al. (1992), the use of transgenic plants for antigen production has received further experimental support from several other groups, who have reported the expression of different antigens using this methodology (Daniell et al., 2009; Floss et al., 2007; Rybicki, 2009; Tiwari et al., 2009). The main problem of this system consisted in the scarcity of the expressed protein, which were often inadequate for commercial development. However, adequately expressed antigens have advanced into clinical trials in a few cases (Aviezer et al., 2009; McCormick et al., 2008). Chloroplasts generally offer higher levels of expression of vaccine antigens; however, glycoproteins cannot be expressed in chloroplast in their glycosylated form.

Stably integrated nuclear transgenes typically yield relatively low concentrations of the recombinant protein, with few examples reporting expression levels higher than 1% TSP (total soluble protein) (Moravec et al., 2007; Nochi et al., 2007; Oszvald et al., 2008; Zhang et al., 2006), neither of them using transgenic alfalfa. Alfalfa is the highest yielding of the temperate forage legumes, has no close relatives and does not contain known mammalian toxins, pathogens or allergens. In addition, technology for drying alfalfa is well established. This plant has been chosen by us and other groups to develop subunit vaccines (Dong et al., 2005; Dus Santos et al., 2002, 2005; Legocki et al., 2005; Perez Filgueira et al., 2004; Wigdorovitz et al., 1999).

In this work, we produced APCH-tE2 protein at a level of 0.1% TSP in transgenic alfalfa plants. This was a significant increase in antigen expression in comparison to previous works with alfalfa.

The presence of the APCH signal peptide in the construction, could direct the secretion of the protein by the default pathway to the apoplast, where it would accumulate and remain protected from cellular proteases (Denecke et al., 1990). As It has been reported, the translocation of proteins to the apoplastic space can be detected by their release in roots tissues (Drakakaki et al., 2006). Future experiments will be conducted in order to examine APCH-tE2 protein location in the plant cell.

Genetically modified plants can be grown in large areas without high investment (Yonekura-Sakakibara and Saito, 2006). Therefore, it seems economically available to use transgenic plants for antigen production. In the case of alfalfa, determination of stability among plants produced by vegetative propagation is a critical issue, to be assessed in order to increase the biomass for antigen production. We found that in the plants obtained by vegetative propagation of the selected transgenic alfalfa plants, APCH-tE2 expression remained stable at an average level of 0.3 μg/ml of extract.

Although large biomass can be obtained easily for oral applications (Chia et al., 2011), the original concept of edible vaccines tend to be replaced by “plant-derived vaccine antigens”. There has been an increased focus on using plant-based production systems to generate purified recombinant proteins for vaccines and therapeutics delivered by injection (Daniell et al., 2009; Tiwari et al., 2009). Some plant-based products for human diseases have had clinical trials success (Aviezer et al., 2009; McCormick et al., 2008) and a vaccine candidate for Newcastle disease in chickens was approved by USDA for use in 2006. This emphasizes that plant-produced targets can progress along the regulatory path (Daniell et al., 2009).

Purification technology is essential if plant-derived vaccines are planed to be administered by injection, and the development of low cost purification methods is important for commercial success. There are few reports for the purification of antigens for veterinary subunit vaccines from transgenic plants (Ashraf et al., 2005; Koya et al., 2005; Nemchinov et al., 2006; Perez Filgueira et al., 2004) all of them on a small scale.

In this work we have standardized an aqueous two phase partitioning system for antigen production. This system turned out easy to execute, was of low cost and high recovery of the recombinant protein making this methodology feasible for downstream processing on a large scale. A mature alfalfa plant can allow the harvest of 50 g of leaf biomass every 21 days. After processing by ATPS a total amount of 80 μg of APCH-tE2 can be produced. This represents 27 doses of vaccine (3 μg APCH-tE2/dose) to be administrated to cattle.

Immunogenicity studies were conducted both in an experimental model and in the natural host. The high correlation observed between bovine and guinea pig models indicated that both models might be useful to monitor BVDV vaccines (Fernandez et al., 2009).

Immunization of guinea pigs with APCH-tE2 produced in alfalfa evoked an antibody response similar to that obtained with APCH-tE2 produced in mammal cells, thus demonstrating that the protein expressed retained its immunogenic properties.

A recent report (Nelson et al., 2012) showed the expression of an tE2 protein obtained from transgenic tobacco plants. In this case a significant larger dose (20 μg/dose) was used to immunized guinea pigs, obtaining neutralizing Ab titers lower than those reported in this work. This discrepancy might be associated to the presence of APCH fuse to tE2, which could enhance the immune response.

The level of NAb induced by vaccination is important for evaluating BVDV vaccine efficacy since it has been reported that titers of NAb higher than or equal to 2 are critical for protection (Bolin, 1995).

A few BVDV recombinant vaccines have been evaluated in cattle, neither of them produced in transgenic plants (Ferrer et al., 2007; Kweon et al., 1997; Rosas et al., 2007; Thomas et al., 2009). We have expressed tE2 in mammalian

cells and immunized cattle with 2 μg of the recombinant protein secreted to the supernatant. After challenge, 6 out of 8 animals vaccinated with E2t showed complete virological protection (Pecora et al., 2012).

In this experience, vaccinated animals that seroconverted after immunization showed high titers of NAb at the time of challenge. Post challenge, NAb titers continued to increase in all vaccinated animals, while calves of the control group remained seronegative until 15 days post challenge. It is important to mention that after vaccination none of the immunized animals showed local reactions or adverse effects.

Total reduction in PMBC-associated viremia was observed in calves immunized with 3 μg of APCH-tE2 produced in alfalfa. On the other hand, 1 calf from the group that received 1.5 μg of APCH-tE2 and 1 calf from the BVDV group shed detectable virus, while virus could be isolated from all animals of the unvaccinated group. Prevention of viremia is a critical point when testing BVDV vaccine efficacy since viremia in a pregnant animal is likely to result in vertical transmission of virus to the fetus.

The results support the conclusion that APCH-tE2 protein expressed in transgenic plants resembles many of the properties of the native viral protein in terms of both antigenicity and immunogenicity as analyzed in the guinea pig model. The same results were obtained in the natural host showing complete virological protection after challenge.

5. Conclusion

This work provides data supporting the feasibility of producing reliable vaccines in plants as an attractive and inexpensive alternative to conventional fermentation systems for vaccine production. Results in the natural host strongly suggest that the plant based BVDV presented a similar performance to a inactivated whole virus vaccine.

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