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Evangelina Gómez, María Soledad Lucero, Silvina Chimeno Zoth, Juan Manuel Carballeda, María José Gravisaco, Analía Berinstein*

- IBDV's VP2 was expressed in *N. benthamiana* retaining its immunogenicity.
- Immunized chickens produced humoral immune response.
- Intramuscular inoculation of chickens elicits neutralizing antibodies.

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Transient expression of VP2 in *Nicotiana benthamiana* and its use as a plant-based vaccine against Infectious Bursal Disease Virus

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ABSTRACT

Infectious Bursal Disease Virus (IBDV) is the etiological agent of an immunosuppressive and highly contagious disease that affects young birds. This disease causes important economic losses in the poultry industry worldwide. The VP2 protein has been used for the development of subunit vaccines in a variety of heterologous platforms. In this context, the aim of this study was to investigate VP2 expression and immunogenicity using an experimental plant-based vaccine against IBDV. We determined that the agroinfiltration of *N. benthamiana* leaves allowed the production of VP2 with no apparent change on its conformational epitopes. Chickens intramuscularly immunized in a dose/boost scheme with crude concentrated extracts developed a specific humoral response with viral neutralizing ability. Given these results, it seems plausible for a plant-based vaccine to have a niche in the veterinary field. Thus, plants can be an adequate system of choice to produce immunogens against IBDV.

1. Introduction

Infectious Bursal Disease Virus (IBDV) is the etiological agent of a highly contagious immunosuppressive disease that affects young birds. Infectious Bursal Disease occurs worldwilde and causes important economic losses in the poultry industry. IBDV is a non-enveloped icosahedral bisegmented double-stranded RNA virus, which is member of the *Birnaviridae* Family. The virus is classified as Serotype I and II but only Serotype I is pathogenic in chickens [1]. The virus infects and destroys IgM-bearing B-lymphocytes in the bursa of Fabricious; which results in immunosuppression [2,3] and T cells (CD4+ and CD8+) infiltration into this organ [4].

Current vaccination with inactivated and live-attenuated vaccines induces immunity in the flock against virulent viruses. Conventional vaccines have a number of disadvantages because of their viral nature. For instance, Live-attenuated vaccines can revert to virulence by the recombination of RNA segments [5]. They also produce a state of immunosuppression in young chickens. Even though this state makes animals susceptible to other diseases, this susceptibility is for a short period and animals can recover. Most importantly, these vaccines are inefficient in protecting birds from very virulent strains of IBDV [6,7]. Moreover, inactivated vaccines are costly and less effective, and they are typically used for boosters of layer hens. Consequently, there is a genuine need to replace the conventional virus-based vaccines by new ones with higher efficacy and fewer side-effects. In this sense, VP2 has been used for the development of subunit vaccines in a variety of heterologous systems. For instance, there are reports of heterologous systems using recombinant fowlpoxvirus [8], herpesvirus [9–12], adenovirus [13,14], baculovirus [15,16], *Escherichia coli* [17], *Pichia pastoris* [18] and plant virus [19]. In addition, DNA vaccines have been obtained [20,21] and VP2 expression and immunogenicity has been reported in transgenic *Arabidopsis thaliana* [22] and rice [23].

Since the past two decades plants have been considered attractive candidates for the production of vaccine antigens to control human and veterinary diseases. It is well documented that antigens expressed *in planta* are capable of inducing protective response when administered by oral or parenteral routes. Thus, this system is very promising as an alternative to produce subunit vaccines. Plant expression systems for foreign protein production have been based on stable and transient transformation. Currently, transient approaches are at the cutting edge of plant production system mainly because the process to obtain the recombinant antigen is

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faster and the yields of recombinant protein are generally higher compared to the stable transformation.

Transient expression can be achieved by tissue infiltration of recombinant *Agrobacterium tumefaciens*, systemic infection of recombinant plant viruses or delivery of viral replicons into the host tissue. It is a simple and useful tool for selecting suitable genetic constructions, which also gives enough material to test the immunogenic properties of the product without the need of purification [24].

In this context, the objectives of the present study were to assess the transient expression of VP2 in plants and to investigate if the recombinant immunogen can be used as a plant-derived vaccine against IBDV. The results obtained in this study may provide further foundation for the development of a new subunit vaccine against IBDV using plants as a platform.

2. Materials and methods

2.1. Virus

Dr. Delamer (Empresa Delamer S.R.L., Argentina) kindly provided the Argentinian field isolate LD-847-04 of IBDV and the classical strain LZD seed of the same virus. LD-847-04 was used to amplify the VP2 coding region and LZD was used for challenge experiments. The virus seed was cultivated in chicken embryo fibroblast (CEF) primary cell culture.

The IBDV vaccine from Laboratorios Inmuner, Argentina (Gumboro LZD Inmuner), was used to vaccinate positive control groups following the manufacturer's instructions.

2.2. Genetic engineering of the expression vector

IBDV RNA extraction and retrotranscription were performed using standard procedures. The coding region of the mature VP2 (1323 bp) was amplified with primers containing NotI and BglII restriction sites (underlined): (1) forward: GCGGCCGCTATGACAAACCTG; (2) reverse: AGATCTGCTCCTGCAATCTTCAGG. The nucleotide sequence comparison of VP2 (Gene bank accession number: JF965438) with the public database was performed using the program BLAST. The comparison resulted in 99% of identity with the very virulent strain 94268 (Gene bank accession number: AY333088.1). VP2 gene was cloned under the rubisco small subunit promoter and the transcription termination signal into the commercial 1.1tag vector (IMPACTVECTOR™, Wageningen UR, Netherlands). This plasmid allows the expression of VP2 fused to c-myc and his tags. The expression cassette was subcloned into the binary vector pBINPLUS (IMPACTVECTOR™, Wageningen UR, Netherlands), which provides right and left borders for nuclear integration (Fig. 1).

The resulting expression vectors were then introduced into *A. tumefaciens* strain GV3101 by electroporation.

2.3. Transient expression of VP2

Transient expression was performed by infiltrating *Nicotiana benthamiana* leaves with a suspension of recombinant bacteria. A construction harboring the green fluorescent protein (GFP) was added as a negative control. The agroinfiltration procedure was conducted as previously described [24]. The infiltrated leaves were harvested 4 days postinoculation and grounded in liquid nitrogen. Subsequently, 3 volumes of chilled extraction buffer (PBS-Tween containing 2 mM Phenylmethyl-Sulfonyl Flouride) were added. After an incubation of 30 min on ice, samples were centrifuged for 15 min at 18000 × *g* and filtered through gauze. The supernatant was twofold concentrated in a centrifugal filter unit (cut off: 30 kDa, Ultracel® YM-30, MILLIPORE™, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, IRL) and samples were kept at −80 °C until use.

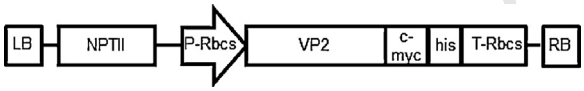

Fig. 1. Schematic representation of the binary vector. NPTII, expression cassette encoding for the kanamycin resistance; P-Rbcs and T-Rbcs, promoter and transcription terminator of rubisco; c-myc and his, tags fused to the VP2 c-terminal; LB and RB, left and right borders, respectively.

2.4. Detection and quantification of the recombinant protein

VP2 expression was analyzed by Western blot assays. Briefly, extracted proteins were separated in 10% SDS-PAGE and blotted into nitrocellulose membrane. Proteins were identified using an anti-VPX/VP2 rabbit polyclonal antibody, kindly provided by Dr. José Rodriguez (Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología/CSIC, Spain), and a monoclonal anti c-myc antibody (Zymed®, Invitrogen, Carlsbad, USA). For protein quantification, we performed a standard curve of bovine serum albumin (BSA). BSA and samples were subjected to SDS-PAGE and VP2 amounts were estimated after coomassie brilliant blue staining. Total soluble proteins (TSP) were determined with a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

2.5. Animals

Embryonated eggs laid by specific pathogen free White Leghorn chickens were purchased from Instituto Rosenbusch S.A. (CABA, Argentina) and hatched in an automatic incubator (Yonar, CABA, Argentina). Chickens were kept in individual cages with food and water *ad libitum*. All procedures were approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE–CICVyA–INTA).

2.6. Experimental vaccine

Each animal received 200 μl of a concentrated crude plant extract containing approximately 12 μg of VP2, emulsified with an equal volume of Freund's adjuvant and 1% total volume of Tween 40. Complete adjuvant was used for the first immunization and incomplete adjuvant thereafter.

2.7. Immunization scheme and challenge

Five chickens of 18 days of age were randomly assigned to the groups. Intramuscular (i.m.) injections were given in pectoral and leg muscles with: plant extracts containing VP2 (group 1), control plant extract containing GFP as a non-related antigen (group 2) or a drop of 50 μl of the IBDV commercial vaccine (group 3). A prime/boost scheme was performed with a boost at 0, 22 and 35 days post first immunization (dpi).

All animals were weekly bled by the wing vein. Eighteen days after the last boost (53 dpi), chickens were challenged by oral inoculation with 500 μl of the classical IBDV strain LZD (6934 $TCID_{50}$/ml). Five days later (58 dpi) animals were euthanized and bursae were removed for lymphocyte isolation and flow cytometry analysis.

2.8. Antibody response against IBDV

Plasma samples of the immunized chickens were weekly evaluated for the presence of specific antibodies against IBDV with a commercial kit (cat No. 99-09260, IDEXX Laboratories, Inc., USA).

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Titers were calculated following the manufacturer's instructions and a value above 396 was considered positive.

2.9. Seroneutralization assay

Plasma samples were inactivated for 30 min at 56 °C and serially diluted twofold, from 1/4 to 1/8192, in culture medium (199 1X, HEPES 25 mM, Gibco®, Invitrogen, Carlsbad, USA). Dilutions were incubated with 100 $TCID_{50}$ of IBDV strain LZD for 1 h at 37 °C in 96-well plates (Greiner bio-one, Germany). Subsequently, 100 μl of a cell suspension of 1.5×10^6 CEFs/ml were added to each well. The cell suspension was prepared in 199 medium supplemented with 3% fetal bovine serum (FBS), HEPES and a mixture of antibiotics/antimycotics (Gibco®, Invitrogen, Carlsbad, USA). Cells were cultured at 37 °C, 5% CO_2 for 4 days, when cytopathic effect was observed. Neutralizing antibody titers were calculated as the inverse of the last dilution showing no cytopathic effect.

2.10. Lymphocyte isolation and flow cytometry analysis

For flow cytometry analysis, bursae of euthanized chickens were processed as previously described [25].

Monoclonal antibodies (mAbs) (CD3-SPRD, CD4-PE, CD8α-FITC, CD8β-PE) were purchased from SouthernBiotech (Birmingham, USA). Cell suspensions were analyzed with a BD FACSCalibur Flow Cytometer (BD FACSCalibur™, BD Biosciences, San José, California, USA) and CellQuest software. The lymphocyte gate was defined by the forward/side scatter characteristics of the cells and 30,000 events were analyzed.

3. Results

3.1. Production of VP2 in Nicotiana benthamiana leaves

The optimum time to harvest the agorinfiltrated leaves was set at the fourth day after infiltration, when yields were the highest. Before performing chicken experiments, the expression of the recombinant VP2 was confirmed by western blot, using the anti VPX/VP2 (Fig. 2) and the monoclonal anti c-myc antibodies (data not shown). A specific band corresponding to the mature VP2 was observed at the expected size and the recombinant protein level was of 1% TSP.

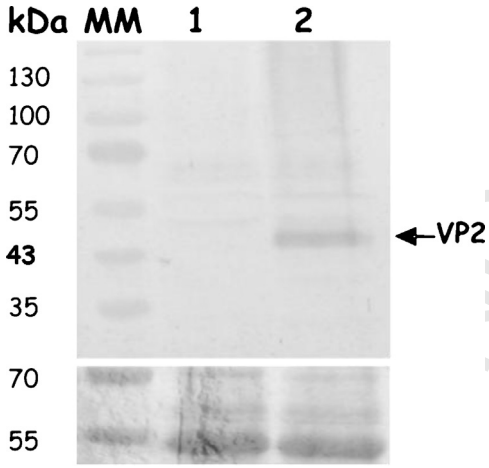

Fig. 2. VP2 transient expression in *Nicotiana benthamiana* plants. Proteins were separated on a 10% SDS-PAGE and recombinant VP2 was identified using an anti-VPX/VP2 antiserum. A negative sample was loaded in lane 1. MM: molecular marker. Gel stained with coomassie blue showing similar quantities of total soluble proteins loaded.

This result confirms VP2 production by plant cells and shows that the heterologous protein was recognized by the specific polyclonal antibody used.

3.2. Immunogenicity of plant-derived VP2 in the natural host of IBDV

To evaluate the plant-derived VP2 as an immunogen for chickens, animals were inoculated with 200 μl of concentrated extract containing 12 μg of recombinant VP2 in a dose/boost scheme. Plasma samples were analyzed for the presence of specific antibodies against IBDV using a commercial ELISA assay. Fig. 3 shows titers of vaccinated animals. All chickens from the experimental group (group 1) mounted a humoral response detected as early as 15 dpi, reaching its highest titers by the end of the experiment. As for the control groups, the negative group (group 2) had undetectable levels of antibodies against IBDV and all animals vaccinated with the commercial vaccine (group 3) showed high titers of specific antibodies. These results indicate that VP2 produced in plants is able to elicit an appropriate immune response in chickens suggesting that the immunogen conserves the antigenic determinants of the wild type protein.

3.3. Plant-derived VP2 elicited an antibody response with neutralizing activity

To determine if the antibodies are capable of neutralizing the virus, a seroneutralization assay was performed using the limit dilution method. Fig. 4 displays the neutralizing titers expressed as the $\log_{10}$ of the inverse of the last dilution without cytopathic effect of samples corresponding to 15, 36 and 48 dpi (2 weeks after each boost). All chickens immunized with the recombinant protein developed a neutralizing response. At first, these titers were low but later on they rose according to the immunization scheme.

These results clearly demonstrate that VP2 produced in plants conserves the neutralizing epitopes. Moreover, the intramuscular route would be an adequate route to generate a systemic response of neutralizing antibodies.

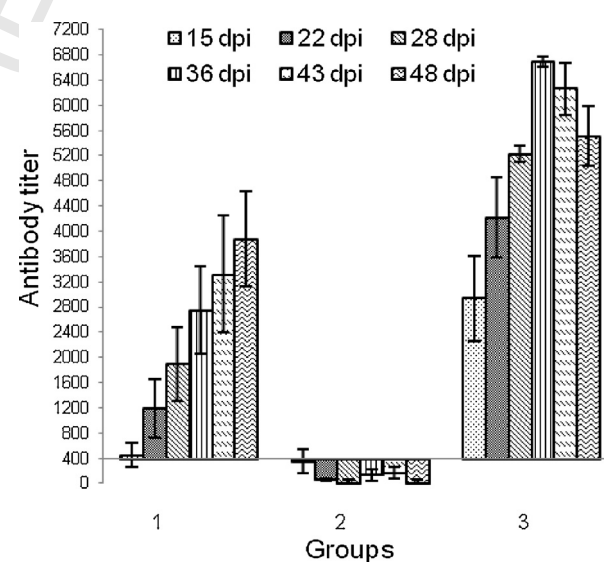

Fig. 3. Evaluation of plasma samples by ELISA. Animals were i.m. vaccinated on 0, 22 and 35 dpi with plant extracts containing VP2 (group 1), control plant extract (group 2) and the commercial vaccine (group 3). Anti-IBDV titers are represented as the mean ± S.D. for each date of the time course. Titers above the cutoff point (396) were considered positive.

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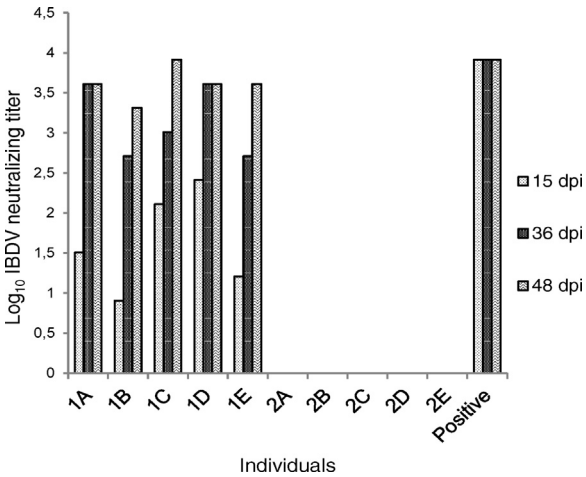

Fig. 4. Neutralizing antibody response of i.m. vaccinated animals. Titers are expressed as the $\log_{10}$ of the inverse of the last dilution that prevented the appearance of cytopathic effect for individual samples. The positive control group titer is also shown.

3.4. Exposure to IBDV after vaccination with plant-derived VP2 resulted in a decreased T-cell infiltration into the bursa

After infection, IBDV replication in the bursa involves an infiltration of T cells into this organ [4,26] and tissue damage. Hence, the frequency of T cells in the bursa of vaccinated animals following a challenge infection could provide an indication of the effectiveness of an experimental immunogen in eliciting a specific immune response and preventing tissue damage. In this context, we investigated the frequency of T cells in the bursa of vaccinated animals after challenge with a high dose of a classical IBDV strain to determine if plant-derived VP2 elicited a protective immune response.

Bursae of vaccinated animals were harvested 5 days after challenge (500 μl of infectious classical IBDV strain containing 6934 $TCID_{50}$/ml) and the lymphocytes from pools of bursae were stained for CD3+, CD4+ and double stained for CD8a+CD8b+. Cell subpopulations were analyzed in the lymphocyte gate defined in a forward/side scatter dot plot. Interestingly, bursae of animals vaccinated with VP2 (group 1) presented fewer infiltrating T cells compared to animals in the control group (group 2). For group 1, 1.44% of the cells corresponded to CD3+, 0.59% to CD4+ and 0.17% to CD8a + CD8b+ cells. In contrast, the control group displayed 4.37% of CD3+, 1.61% of CD4+ and 3.85% of CD8a + CD8b+ cells. Moreover, bursae of the animals immunized with the experimental immunogen showed a normal morphology, while those of the negative control group displayed a yellowish appearance which is typical of IBDV infection (data not shown).

4. Discussion

In this study, we investigated the expression, immunogenicity and protective efficacy of a plant-based vaccine against IBDV. We determined that the agroinfiltration of *N. benthamiana* leaves, a model species for transient expression assays [24], allowed the production of VP2. VP2 has been described as the main immunogenic protein of IBDV. It possesses the virus neutralizing epitopes and is responsible for cell tropism and virulence [27–31]. We also observed that chickens intramuscularly immunized in a prime/boost scheme with crude concentrated extracts developed a specific humoral response with viral neutralizing capacity. This response was observed as early as 15 days after the first inoculation. Consistent with previous findings [32,33], we found that VP2 was able to mount a protective immune response. The total IgG titer of the VP2 immunized animals was significantly lower than those of the positive control animals immunized with the commercial IBDV vaccine (Fig. 3). However, no differences in the neutralizing antibody titers were observed between the two groups (Fig. 4). The ELISA assay measures antibodies against the whole virus, while the neutralization assay evaluates the capability of the antibodies to inhibit virus infection. Therefore, our findings, strongly suggest that anti-VP2 antibodies are responsible for virus neutralization and that VP2 expressed in plants conserves the correct immunogenic folding.

In this study, we analyzed the ability of the immunogen to prevent T-cell infiltration into the bursa as a parameter of protection against an infectious virus. We observed a decrease in the frequency of T-cell infiltration into the bursa as a consequence of plant-derived VP2 immunization (from 2.7 to 22.6 times fewer than the control group). These results suggest that the humoral response prompted by the recombinant immunogen neutralizes totally or partially the entrance of IBDV.

In earlier studies VP2 was also assessed as an immunogen by the oral route. Soluble VP2 expressed in *A. thaliana* was given to animals with a scheme of five oral doses at intervals of 3 days (with 5.5 μg of VP2). This scheme mounted an antibody response and 80% of protection against challenge [34]. Furthermore, fasted chickens fed with 5 g of rice seeds expressing VP2 produced neutralizing antibodies against IBDV and were protected (83.33%) against challenge [23]. In the same study, the authors observed a dose-dependent correlation. Both studies have demonstrated that VP2 is resistant to gut degradation, based on the fact that it invoked an immune response.

A recent study showed the capability of purified bamboo mosaic virus displaying the loop P_{BC} of VP2 (fused to the capsid protein) to elicit specific humoral responses and protection against IBDV [19]. As far as we know, however, the present study is the first report in which the full length VP2 is transiently expressed in plants using the agroinfiltration technique.

The potential use of plants for the production of recombinant vaccines has been previously tested for other important poultry diseases. For instance, several proteins of the chicken anemia virus (CAV) have been transiently expressed in *N. benthamiana* by recombinant PVX virus or binary vectors [35]. Although CAV proteins were successfully expressed as fusion proteins with GFP, the levels of expression varied considerably between proteins. In the research just mentioned, the signal of GFP: VP1 was too low to be detected using Western blot analysis. However, 1.2% of TSP was estimated for GFP:VP2 and 2.6% of TSP for GFP:VP3. Unfortunately, immunogenicity was not explored in this study. The authors stated that protein levels need to be improved before exploiting them as oral vaccines. Another study, although not strictly conducted to obtain poultry vaccines, describes the expression of HA of a highly pathogenic avian influenza virus (H5N1) in tobacco by means of agroinfiltration [36]. Four chickens were vaccinated with the plant immunogen in its full length form or a truncated variant formulated with Incomplete Freund Adjuvant. Although HA analysis revealed that H5 had a proper conformation, the HI titers obtained for the two positive animals vaccinated with the truncated form were relatively low. Besides, Kalthoff et al. [37] obtained a yield of 20–30 mg of purified rHA0 per kilogram of fresh leaf biomass using the magnICON provector system. Immunized chickens with 50 or 100 μg of antigen combined with different adjuvants developed a humoral specific response and had a survival rate of 89–100%. Kanagarajan et al. [38] demonstrated the transient expression in *N. benthamiana* of rHA0 from a low pathogenic avian influenza virus with a yield of 0.2 g of purified protein per kg of leaf fresh weight. In this study, no animal experiment was conducted. Antigens of *Eimeria tenella* have been also transiently expressed by agroinfiltration in tobacco leaves with yields of 25 mg/kg of fresh biomass [39]. In our study, VP2 represented just 1% of TSP. Although this level could be

optimized, a two-fold concentrate of the extract (12 μg of protein) applied in three doses was clearly enough to induce a neutralizing antibody response in chickens.

Taken into account the disadvantages of the commercial live-attenuated and inactivated vaccines, a plant-based subunit vaccine represents a viable alternative in the veterinary field [40–42]. Plants could be an excellent choice to produce veterinary antigens, since they are potentially both very economical and infinitely scalable. Moreover, we propose that transient expression is a very promising strategy to produce the main immunogenic protein from IBDV to further obtain a subunit vaccine.

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