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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.

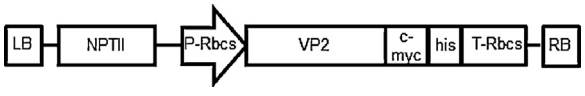

Fig. 1. Schematic representation of the binary vector. NPTII, expression cassette encoding for the kanamycin resistance; P-Rbcs and T-Rbcs, promoter and transcription terminator of rubisco; c-myc and his, tags fused to the VP2 c-terminal; LB and RB, left and right borders, respectively.

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).