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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Functional characterization of a plant-produced infectious bursal disease virus antigen fused to the constant region of avian IgY immunoglobulins

Emile Rage[1] · Charifa Drissi Touzani[2] · Carla Marusic[1] · Chiara Lico[1] · Thomas Göbel[3] · Alessio Bortolami[4] · Francesco Bonfante[4] · Anna Maria Salzano[5] · Andrea Scaloni[5] · Siham Fellahi[2] · Mohammed El Houadfi[2] · Marcello Donini[1] · Selene Baschieri[1]

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Abstract

Infectious bursal disease virus (IBDV) is the cause of an economically important highly contagious disease of poultry, and vaccines are regarded as the most beneficial interventions for its prevention. In this study, plants were used to produce a recombinant chimeric IBDV antigen for the formulation of an innovative subunit vaccine. The fusion protein (PD-FcY) was designed to combine the immunodominant projection domain (PD) of the viral structural protein VP2 with the constant region of avian IgY (FcY), which was selected to enhance antigen uptake by avian immune cells. The gene construct encoding the fusion protein was transiently expressed in *Nicotiana benthamiana* plants and an extraction/purification protocol was set up, allowing to reduce the contamination by undesired plant compounds/proteins. Mass spectrometry analysis of the purified protein revealed that the glycosylation pattern of the FcY portion was similar to that observed in native IgY, while in vitro assays demonstrated the ability of PD-FcY to bind to the avian immunoglobulin receptor CHIR-AB1. Preliminary immunization studies proved that PD-FcY was able to induce the production of protective anti-IBDV-VP2 antibodies in chickens. In conclusion, the proposed fusion strategy holds promises for the development of innovative low-cost subunit vaccines for the prevention of avian viral diseases.

Keywords CHIR-AB1 · Glycosylation · IgY · IBDV · Molecular farming · Veterinary vaccine

Introduction

Infectious bursal disease, also known as Gumboro disease, is a worldwide distributed viral infection of chickens that causes the inflammation and atrophy of the bursa of Fabricius (BF), the lymphoid organ where the differentiation and maturation of B lymphocytes occur in birds (Ingrao et al. 2013). The resulting immunosuppression increases the sensitivity of flocks to bacterial, protozoal, and other viral diseases, with consequent important economic losses for the poultry industry (Sharma et al. 2000; Mahgoub 2012). Infectious bursal disease virus (IBDV) is extremely contagious for young chickens, with the highest susceptibility reached between 3 and 6 weeks of age, when BF is at a critical developmental stage (Eterradossi and Saif 2013). Two IBDV serotypes exist but only the serotype 1 is pathogenic (McFerran et al. 1980). Different strains of this serotype have been described and

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✉ Marcello Donini
marcello.donini@enea.it

[1] Laboratory of Biotechnology, ENEA Casaccia Research Center, Rome, Italy

[2] Unité de Pathologie Aviaire, Département de Pathologie et Santé Publique Vétérinaire, IAV Hassan II, Rabat, Morocco

[3] Department of Veterinary Sciences, LMU Munich, München, Germany

[4] Division of Comparative Biomedical Science, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy

[5] Proteomics & Mass Spectrometry Laboratory, ISPAAM, National Research Council, Napoli, Italy

classified as classical or very virulent (vvIBDV). The vvIBDV strains, which show stronger severity of symptoms and higher mortality rates compared with classical strains, emerged in Europe and Japan at the end of the 80s. Nowadays, vvIBDV strains have spread all over the world and represent a major threat to poultry farming (Mahgoub 2012).

IBDV is a non-enveloped virus with a T = 13 icosahedral capsid belonging to the family of *Birnaviridae* and its genome consists of two linear double-stranded RNA segments designated A and B with a total length of 6 kilobase pairs (kbp) (Müller et al. 1979). The segment A is about 3.2 kbp long and contains two partly overlapping open reading frames (ORF). The larger ORF encodes a polypeptide (110 kDa) that is autocatalytically cleaved in 3 proteins: the structural components VP2 (42 kDa) and VP3 (32 kDa), and the serine protease VP4 (28 kDa) (Berg 2000; Coulibaly et al. 2005). VP2, the most abundant structural protein, is composed of three domains, namely the shell (S), the base (B), and the projection domain (PD), the latter being involved in the elicitation of neutralizing antibodies (Fahey et al. 1989).

There are no specific therapeutic treatments for IBDV infection, and disease diffusion is mostly controlled with vaccination programs based on inactivated or live-attenuated viruses (Müller et al. 2012). Inactivated vaccines need to be injected several times together with adjuvants, contributing to increase labor-intensity and costs, while attenuated vaccines may induce bursa lesions and immunosuppression and present a risk of reverting to virulence (He et al. 2009). In order to overcome these issues, novel recombinant subunit vaccines are under development, which offer several advantages such as the cost-effectiveness of the production strategies, safety, and the possibility to differentiate infected from vaccinated animals (DIVA) (Hasan et al. 2016). Recombinant IBDV subunit vaccines are mainly based on the production of the major structural/immunogenic VP2 protein using different expression systems, such as *Escherichia coli* (Jiang et al. 2016), insect cells (Ge et al. 2015), and yeasts (*Pichia pastoris*) (Taghavian et al. 2013).

Plant expression systems have been extensively used for the production of recombinant antigens for veterinary applications, including IBDV vaccine development, mainly because of their safety and potential low manufacturing costs (Topp et al. 2016). Several plant-based platforms have been used for the production of VP2, such as transgenic rice and *Arabidopsis thaliana* (Wu et al. 2007; Wu et al. 2004), or transiently transformed *Nicotiana benthamiana* (Gómez et al. 2013; Richetta et al. 2017). In all of these studies, the plant production of the full-size VP2 protein was verified as well as the possibility to use it in vaccination studies through parenteral or oral administration to chickens. In some cases, a mild efficacy in the protection of animals from IBDV challenge was demonstrated.

The aim of this work was to produce in plants an IBDV chimeric antigen based on the fusion of the PD of VP2 to the constant region of avian IgY immunoglobulins (herein indicated as FcY). PD was selected because it contains two hydrophilic loops carrying important neutralizing conformational epitopes (Letzel et al. 2007), and FcY as fusion moiety to potentiate the immune response against the antigen by improving targeting and uptake by receptors such as FcRY and CHIR-AB1 (He and Bjorkman 2011; Viertlboeck and Göbel 2011). These receptors are involved in the transfer of IgY across the avian yolk sac and in the uptake by chicken immune cells (B lymphocytes, macrophages, and Natural Killer cells) and may be able to transfer IgY across the mucosal epithelial cells similarly to mammalian Fc receptors (FcR) (Tesar et al. 2008; Yoshida et al. 2004). Therefore, PD-FcY could be potentially used as an efficient immunogen for the activation of mucosal immunity. Besides the immunological aspects, this fusion strategy presents also the advantage of using the Fc region as a stabilizing partner to increase expression levels and as a tag for recombinant protein detection and purification (Lu et al. 2012).

The PD-FcY chimeric antigen was transiently expressed in *N. benthamiana* plants. Thus, optimized conditions for downstream processing were defined and preliminary structural and functional studies, both in vitro and in vivo, were performed to lay the foundations for a future application in the veterinary field.

Materials and methods

vvIBDV strain isolation, VP2 sequencing, and anti-IBDV antibody production

A field isolate of vvIBDV was used to amplify the VP2 coding region. vvIBDV L1/08 was propagated in 6-week-old specific-pathogen-free (SPF) chickens, housed in high-efficiency particulate air-filtered poultry isolators at the facilities of the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). Bursae of Fabricius were collected from birds 3 days post-infection, homogenized in phosphate-buffered saline (PBS) at a 10% w/v ratio, and centrifuged at 4500×*g* for 30 min. To remove the lipid fraction, samples were treated with an equal volume of chloroform and the supernatant was collected after centrifugation. Purification of the virus was made by 20–60% (w/v) discontinuous sucrose gradient centrifugation in a SW28 rotor (Beckman Coulter, CA, USA) at 25,700 rpm for 3.5 h. Purified virus was inactivated by dialysis against formalin (0.1%) and used to immunize a rabbit for the production of a polyclonal serum against vvIBDV. For the production of a chicken polyclonal serum against vvIBDV, formalin-inactivated bursal homogenates were inoculated in 6-week-old SPF chickens.

Plant expression constructs

The *pd-fcy* gene (GenBank Accession Number MK908400) was synthetically constructed by fusing the nucleotides encoding the amino acid sequence 10–148 of the IBDV VP2 protein (GenBank Accession Number ABF93430.1) corresponding to the PD with those encoding the amino acid sequence 167–504 of a chicken IgY (GenBank Accession Number S00390) corresponding to the Fc domain (Fig. 1a) (Parvari et al. 1988). The fusion gene was preceded by the sequence encoding the secretory signal peptide of an embryonic mouse immunoglobulin heavy chain-gene (L) (GenBank Accession Number ANN23957.1; amino acids 1-17) (Villani et al. 2009). The sequence of the synthetic gene was codon-optimized according to the codon bias of *N. benthamiana* (http://www.kazusa.or.jp/codon) using the GENEius software (Eurofins Genomics, Ebersberg, Germany). The synthetic sequence (Eurofins Genomics) was excised from pEX plasmid by digestion with *Bam*HI and *Xma*I restriction enzymes, and inserted into similarly digested pGEM-NOS plasmid (Lombardi et al. 2009) to be then transferred, together with the Nopaline synthase terminator (NOSter), into the binary vector pBI-Ω (Marusic et al. 2007) using *Bam*HI/*Eco*RI restriction sites. In this vector, gene expression is under the control of the constitutive Cauliflower Mosaic Virus 35S promoter (35SCaMV), the Ω translational enhancer sequence from Tobacco Mosaic virus and the NOSter sequence.

Transient expression in *N. benthamiana* plants

Agrobacterium tumefaciens (LBA4404) (Thermo Fisher Scientific, Rockford, IL, USA) harboring either the plant expression vector with the PD-FcY construct or the p19 construct (p19 silencing suppressor gene from the Artichoke Mottled Crinkle Virus, AMCV) (Lombardi et al. 2009) were grown separately in Luria-Bertani medium supplemented with appropriate antibiotics. Bacterial cultures were then pelleted at 4000×*g* for 10 min and resuspended in leaf infiltration buffer [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $MgCl_2$, pH 5.8]. The *A. tumefaciens* suspensions were mixed to a final optical density at 600 nm (OD_{600}) of 0.4 each, before being used for either pressure infiltration with a needleless syringe (small-scale production) or vacuum infiltration (large-scale production) of *N. benthamiana* leaves. The large-scale production was made submerging 6-week-old plants grown under standard conditions (24 °C, 16 h light/8 h dark) into the bacterial suspension, applying a 10 mmHg vacuum and releasing it to provoke infiltration of the leaves. Leaves were collected 4 to 10 days post-infiltration (d.p.i) for the validation of PD-FcY expression, or at 6 d.p.i for PD-FcY purification. The negative control was obtained by infiltrating plants only with *A. tumefaciens* carrying the p19 construct.

Protein extraction and PD-FcY purification

Protein extracts for the analysis of agroinfiltrated leaves were obtained by grinding 200 mg of tissue in liquid nitrogen and by homogenization with Ultraturrax T25 (IKA-Werke, Staufen, Germany) in PBS pH 7.5 supplemented with a protease inhibitor cocktail (cOmplete™; Roche, Mannheim, Germany) in a 1:2 weight/volume ratio. The extracts were centrifuged at 20,000×*g* for 20 min, the supernatants were recovered, and the total soluble protein (TSP) content was determined by Bradford colorimetric assay as specified by the manufacturer (Bio-Rad Protein Assay, Hercules, CA, USA). Intercellular fluids (IF) were obtained by infiltrating the leaves after collection with PBS using a vacuum infiltration device. After vacuum release, leaves were gently paper-dried and placed in 50-ml Falcon tubes perforated at the bottom to allow liquid flow through. The tubes were placed in 250-ml tubes and centrifuged at 800×*g* for 10 min to obtain clear fluids corresponding to IF that were directly analyzed.

For PD-FcY purification, leaves were ground in liquid nitrogen in the presence of 4% w/w polyvinylpolypyrrolidone (PVPP) and mixed 1:2 with acidic extraction buffer (20 mM sodium phosphate, 1% w/v ascorbic acid) and homogenized using Ultraturrax T 25. After Miracloth paper filtration and centrifugation at 20,000×*g* for 20 min; the supernatant was added with 4% w/v PVPP, incubated 2 h at room temperature (r.t.), and then centrifuged at 10,000×*g* for 10 min. The supernatant was then adjusted to pH 7.5 and centrifuged again at 20,000×*g* for 20 min. A first precipitation step was performed by adding to the supernatant ammonium sulfate up to 15% saturation, at 4 °C, for 1 h, with gentle agitation. The solution was then centrifuged at 10,000×*g* for 20 min, and the supernatant was added with ammonium sulfate up to 50% saturation for the second precipitation step, and gently stirred 1 h at 4 °C. After a further centrifugation at 10,000×*g* for 20 min, the pellet was resuspended in extraction buffer (20 mM sodium phosphate, pH 7.5) in the case of the Ligatrap IgY column or in PBS in the case of the GenScript IgY resin. Extracts were then filtered through a 0.45-μM PES membrane before purification.

The clarified extract was passed through the Ligatrap IgY column based on peptoid ligands that specifically bind IgY (Ligatrap™ Technologies, Raleigh, NC, USA) and elution from the column (0.5 ml fractions) was performed using 0.1 M sodium acetate pH 4.0, and the positive selected fractions were pooled, dialysed in PBS pH 7.2, and concentrated using Vivaspin 20 columns (PES membrane, 50,000 molecular weight cut-off) (Sartorius, Stonehouse, UK) following the manufacturer's instructions. TSP content in the extracts or column eluted samples was determined by Bradford colorimetric assay (Bio-Rad, Hercules, CA, USA).

In the case of the Genscript IgY resin protocol, clarified extract was incubated with the resin (GenScript, Piscataway,

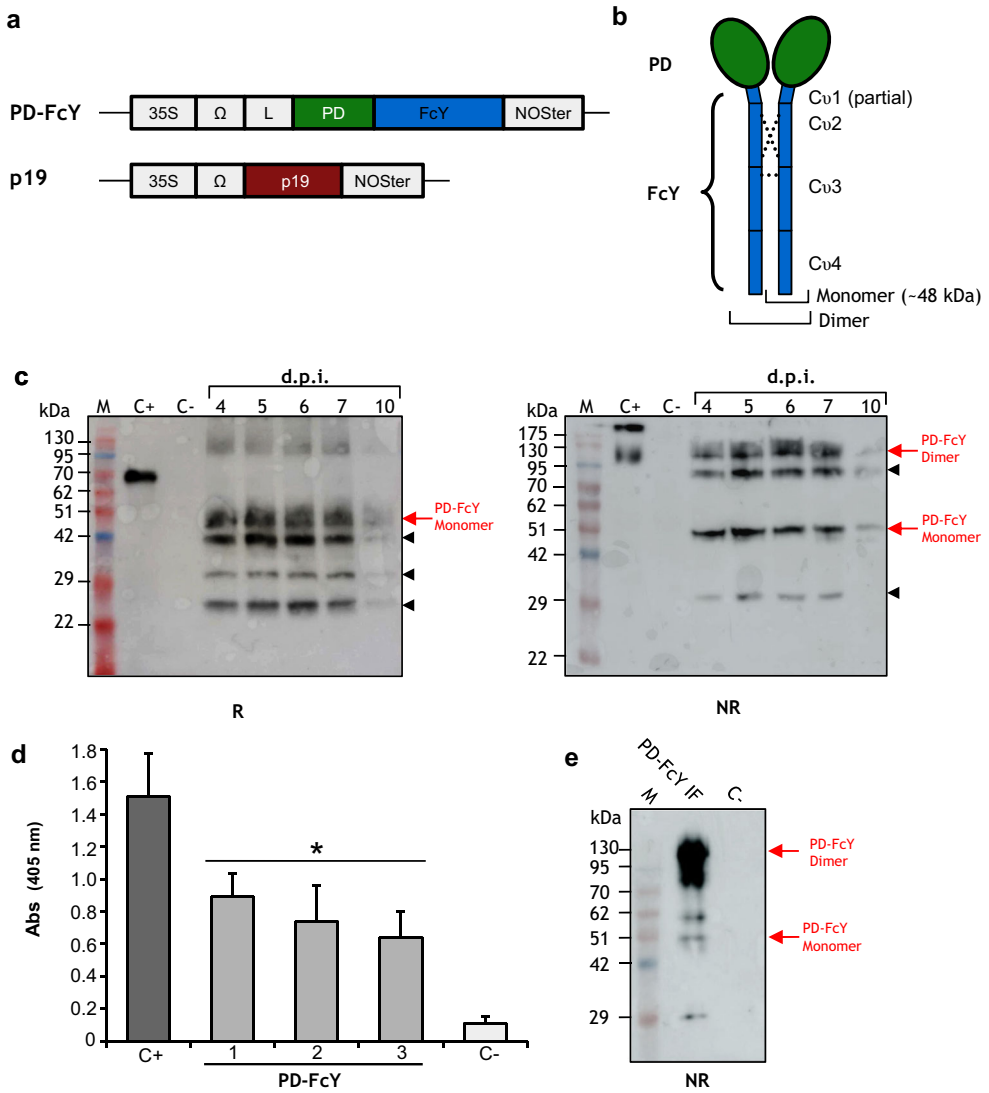


Fig. 1 Schematic representation of plant expression constructs and analysis of PD-FcY expression in *N. benthamiana* leaves. **a** Schematic representation of the PD-FcY and the Artichoke Mottled Crinkle Virus (AMCV) p19 constructs used for plant transient expression. Gene expression was under the control of the Cauliflower Mosaic Virus 35S promoter (35S), the translation enhancer sequence of Tobacco Mosaic Virus (Ω), and the Nopaline synthase terminator sequence (NOSter). L, sequence encoding the secretion signal peptide from the heavy chain of an embryonic mouse immunoglobulin. **b** Schematic illustration of the PD-FcY dimer. The approximate molecular mass value of the monomeric form is indicated in brackets. Cυ 1 → 4: constant region domains of avian IgY. Dotted lines: disulfide bonds. **c** Western blot analysis with an anti-IgY antibody of protein extracts prepared from PD-FcY agroinfiltrated leaves at different d.p.i, separated by 10% SDS-PAGE under reducing (R, left panel) and non-reducing (NR, right panel) conditions. Five micrograms of TSP were loaded in each well. M: molecular weight marker; C−: leaves infiltrated only with *A. tumefaciens* harboring the p19 construct; C+: purified chicken IgY (100 ng). Black arrow heads: putative PD-FcY degradation products. **d** Double antibody sandwich ELISA (DAS) of PD-FcY plant extracts. Capture antibody: chicken anti-IBDV serum. C+ : inactivated IBDV virus (0.5 μl); 1, 2, 3: PD-FcY plant extracts (100 μl, 50 μl, 25 μl, respectively); C−: p19 plant extract (100 μl). Detection antibody: rabbit anti-VP2 polyclonal antibody. Values are the mean ± standard deviation (SD) of triplicate wells. *Student's *t* test, $p < 0.05$ for PD-FcY samples versus C−. **e** Western blot analysis of proteins present in the intercellular fluids (IF) of agroinfiltrated leaves at 6 d.p.i. Twenty microliters was separated by 10% SDS-PAGE under non-reducing conditions. C−: IF from leaves infiltrated only with *A. tumefaciens* harboring p19 construct; PD-FcY-IF: IF from leaves infiltrated with *A. tumefaciens* strains harboring PD-FcY and p19; M: molecular weight marker

NJ, USA) at r.t., for 30 min. The resin was recovered by centrifugation at 5000×*g* for 1 min and, after three washes with PBS, proteins were eluted by boiling the resin 5 min in SDS-PAGE sample loading buffer. The supernatant containing the purified product was recovered after centrifugation at 5000×*g* for 30 s.

SDS-PAGE and Western blot analysis

Proteins were separated by 10–12% SDS-PAGE under non-reducing and reducing conditions, the latter was obtained by adding 3% β-mercaptoethanol into the sample loading buffer (50 mM Tris HCl pH 6.8, 2% w/v SDS, 10% glycerol, 0.1%

w/v bromophenol blue). Proteins were transferred to a PVDF membrane (Amersham™ Hybond® P 0.45 μM, GE Healthcare, Freiburg, Germany) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare) for 70 min, at 0.8 mA/cm^2 and 30 V. Membranes were incubated overnight (o.n.) at 4 °C in PBS containing 5% w/v skimmed milk (5% milk-PBS, blocking solution). Purified chicken IgY (AC146, Millipore, Temecula, CA, USA) was used as positive control; as expected, it migrated in SDS-PAGE with an apparent molecular mass of about 70 kDa in reducing conditions, and of about 180 kDa in non-reducing conditions. PiNK plus or BLUelf (GeneDireX, Las Vegas City, NV, USA) were used as pre-stained protein molecular weight (MW) markers. Detection was performed with anti-IgY horseradish peroxidase (HRP)-conjugated antibody (A9046, Sigma-Aldrich, Saint-Louis, MO, USA) diluted 1:2000 in 2% milk-PBS. The ECL SuperSignal® West Pico (Thermo Fisher Scientific, Rockford, IL, USA) and ImageQuant™ LAS 500 system (GE Healthcare) were used for chemiluminescence signal detection. Proteins migrated in SDS-PAGE were stained by o.n. incubation with Coomassie R250 (Sigma-Aldrich), at r.t., before destaining with 25% methanol and 10% acetic acid. Protein quantification was performed by densitometric analysis of the bands on the gel with the ImageQuant TL 7.0 Image analysis software (GE Healthcare) using as standard different concentrations of bovine serum albumin (BSA).

Enzyme-linked immunosorbent assay (ELISA)

Direct ELISA was performed by coating the wells of ELISA MaxiSorp1 plates (NUNC, Roskilde, Denmark) with the antigens for 2 h, at 37 °C. The wells were then blocked with 5% milk-PBS for 2 h, at 37 °C, washed and then incubated with the rabbit anti-IBDV serum diluted 1:500 in 2% milk-PBS, at 4 °C, o.n. After incubation with the secondary goat anti-rabbit HRP-conjugated antibody (31460, Thermo Fisher Scientific), antigen-bound antibodies were revealed using 2,20-azino-di-3-ethylbenz-thiazoline sulphonate (ABTS) (KPL, Milford, MA, USA), and the colorimetric reaction measured with an ELISA reader at 405 nm (TECAN-Sunrise, Groedig, Austria).

Double Antibody Sandwich (DAS)-ELISA was performed by coating the wells of the ELISA plates with chicken anti-IBDV serum diluted 1:50 in 2% milk-PBS, and incubated at 4 °C, o.n. After blocking with 5% milk-PBS for 2 h, at 37 °C, and washes with 0.1% v/v Tween-PBS, the antigens or PBS were added in triplicate into the wells, and incubated at 4 °C, o.n. After the washes, wells were incubated with a rabbit anti-VP2 serum (kindly provided by Prof. J.R. Caston, Centro Nacional de Biotecnología/CSIC, Cantoblanco, Madrid, Spain) (Fernández-Arias et al. 1998) at a 1:1000 dilution in 2% milk-PBS for 2 h, at 37 °C, and then incubated with a goat anti-rabbit HRP-conjugated antibody at a 1:5000 dilution in 2% milk-PBS for 1 h, at 37 °C. The binding was revealed as described before. The presence of IBDV-specific antibodies in the sera of chickens was determined using a commercial ELISA kit (ProFLOK® IBD PLUS ELISA kit, Zoetis, NJ, USA) following the manufacturer's instructions. This kit is routinely used for the development and evaluation of vaccination programs, and is highly specific for the detection of antibodies against IBD recombinant VP2 in classic and variant strains.

Proteomic and glycosylation analysis

The purified fusion protein was separated on a reducing SDS-PAGE (10%) and was Coomassie stained, and bands were excised, triturated, in-gel reduced and S-alkylated, and finally digested with endoprotease Asp-N (Roche, Mannheim, Germany) (Salzano et al. 2013). Peptides were extracted from the gel particles using 5% formic acid/acetonitrile (1:1 v/v), and digest solutions were concentrated and desalted before mass spectrometry analysis by using μZipTipC18 pipette tips (Millipore). Protein digests were analyzed by nanoLC-ESI-Q-Orbitrap MS/MS using a LTQ XL Q-ExactivePlus mass spectrometer equipped with a Nanoflex ion source (Thermo Fisher Scientific) and connected to an UltiMate 3000 HPLC RSLC nano system-Dionex (Thermo Fisher Scientific). Peptides were separated on an Acclaim PepMap RSLC C18 column, 150 mm × 75 μm internal diameter, 2-μm particles, and 100-Å pore size (Thermo Fisher Scientific) as previously reported (Lonoce et al. 2019). Full mass spectra were acquired in the range m/z 375–1500, with nominal resolution 70,000. Fragmentation of parent ions was controlled by a data-dependent scanning procedure over the 10 most abundant ions using 30-s dynamic exclusion. Mass isolation window and collision energy were set to m/z 1.2 and 32%, respectively. NanoLC-ESI-Q-Orbitrap MS/MS data were searched with Proteome Discoverer (v.2.2 0) using MASCOT node (v.2.2.06) (Matrix Science, London, UK) and Byonic™ software (v.2.6.46) (Protein Metrics, Cupertino, USA) against a database containing the sequence of the recombinant PD-FcY and common contaminants (Zhu et al. 2017). Searching parameters were *N*-terminal Asp/Glu as cleavage specificity, allowing also semi-specific cuts and 3 missed cleavages as maximum value, Cys carbamidomethylation as fixed modification, and Met oxidation, *N*-terminal Gln cyclization, Asn/Gln deamidation, and Asn *N*-glycosylation as variable modifications. Mass tolerance values for peptide matches were set to 10 ppm Da and 0.05 Da for precursor and fragment ions, respectively. For glycopeptide identification with Byonic™, a homemade glycan modification database was built up including common biantennary structures and typical plant *N*-linked glycoforms. Score thresholds for accepting peptide and glycopeptide identifications were MASCOT ion score > 30, with a significant threshold $p < 0.05$, and a Byonic™ score > 150. Glycopeptide identifications were manually validated to

finally assign the glycoforms. The relative percentage of the glycoforms was calculated by using the area of the extracted ions for each validated glycopeptide species (choosing the three most intense ions) assuming a comparable ionization efficiency for all of them. Reported relative percentages are mean values of two independent measurements normalized by the total area of the ion chromatogram.

Binding to the avian immune receptor CHIR-AB1

The binding of purified PD-FcY protein was tested as described before using the BWZ.36-CHIR-AB1 cells (Viertlboeck et al. 2007). Briefly, 3×10^5 reporter cells were cultivated for 24 h in 24-well cell culture plates. The cell culture dishes were either left untreated (CHIR-AB1 cells only), treated with 10 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) (positive control), or coated with the specific monoclonal antibody 8D12 (10 μg/ml), a chicken IgY (10 μg/ml), the partially purified PD-FcY protein (50 μg/ml of total purified proteins containing 10 μg/ml of PD-FcY), or a similarly processed p19 plant extract (50 μg/ml of purified plant proteins). The β-galactosidase activity was measured using 130 μl/well chlorophenolrot-β-D-galactopyranoside (CPRG, Roche, Mannheim, Germany) as substrate, and quantified by optical density reading at 575 nm 4 h after incubation. The values of three independent assays were combined to calculate the mean ± SD.

Animal studies

Fifteen specific-pathogen-free (SPF) chickens of 5 days of age were kindly provided by MCI Animal Health Laboratories (Mohammedia, Morocco) and randomly divided into three groups of five animals. All animals were kept at constant temperature range (20–25 °C) and 30–40% relative humidity, with free access to food and water. The first group of chicks was immunized intramuscularly with 120 μl of partially purified PD-FcY (40 μg of total purified proteins containing 8 μg of PD-FcY) emulsified in 80 μl of Montanide ISA 71 VG (Seppic, France) kindly provided by Biopharma Laboratories (Rabat, Morocco). Similarly, the second group was injected intramuscularly with 120 μl of p19 negative control (40 μg of total purified plant proteins) emulsified in 80 μl of adjuvant. The third group was used as a positive control and immunized with a commercial inactivated IBD vaccine (NOBILIS® GUMBORO inac, IBDV D78 strain) (Pharmavet, Morocco). All groups were injected four times at weekly intervals, starting at day 10 post hatch.

At 41 days of age, all groups were challenged via the oculonasal route with a vvIBDV Moroccan strain isolated from broiler chickens in 2017 (Drissi Touzani et al. 2019). The titer of the vvIBDV inoculum was 10^5 EID_{50} (50% embryo infective dose). The clinical signs and mortality were recorded daily during 10 days post-challenge. Blood samples were collected from the wing vein the day before each immunization and at day 41 just before the challenge.

Statistical analysis

Statistical analysis was performed using Student's *t* test on R Software version 3.4.3 (R_Core_Team 2013). p values < 0.05 were considered statistically significant. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com.

Results

PD-FcY expression in plants

N. benthamiana leaves were co-infiltrated with *A. tumefaciens* strains carrying PD-FcY and p19 constructs to produce a secretory version of PD-FcY recombinant protein (Fig. 1a and b). The p19 silencing suppressor protein was used to enhance protein accumulation. Leaves were collected at 4, 5, 6, 7, and 10 days post-infiltration (d.p.i), and the presence of PD-FcY in crude extracts normalized for TSP content (Figure S1) was assessed by SDS-PAGE followed by Western blot (WB) analysis using an anti-IgY antibody (Fig. 1c). Extracts from leaves infiltrated only with *A. tumefaciens* carrying the p19 construct were used as negative control (C−). The results demonstrated that the highest accumulation of PD-FcY into the leaves was reached at 6 d.p.i. Under reducing conditions (Fig. 1c, left panel), the presence of one band at about 48 kDa, corresponding to the expected size of the intact PD-FcY, and of three additional bands with lower molecular mass values (approx. 45, 30, and 22 kDa), probably corresponding to degradation products, was evidenced. The analysis of the same samples under non-reducing conditions (Fig. 1c, right panel) showed the presence of three major bands: the lowest one (migrating at about 48 kDa) corresponded to the monomer, while two other ones (migrating at about 100 and 90 kDa) were probably related to dimers of intact and degraded PD-FcY, respectively (Fig. 1c, right panel). Bands of higher molecular mass values (> 100 kDa) were also present, possibly indicating the formation of multimers. The presence of the intact PD domain in the plant-expressed fusion protein was confirmed by ELISA experiments with a rabbit polyclonal antibody specific for its N-terminal sequence (Fig. 1d). To verify the secretion and correct processing of the fusion protein, also intercellular fluids (IF) of leaves at 6 d.p.i were assayed for the presence of PD-FcY by SDS-PAGE performed under non-reducing conditions followed by WB analysis (Fig. 1e). A protein pattern similar to the one observed in the leaf extract was evidenced, but with

a more intense signal of the bands at higher molecular masses (90 and 100 kDa).

PD-FcY extraction and purification

The extraction/purification protocol of PD-FcY was set up using batches of 20 g of vacuum-agroinfiltrated *N. benthamiana* leaves. To remove undesirable plant compounds (e.g., phenols/pigments) and proteins, the plant tissue was homogenized in an acid buffer containing PVPP and ascorbic acid, adjusting the pH to 7.5 before centrifugation. By using this approach, the supernatant exhibited a clear colorless aspect, and the TSP content was significantly reduced (Figure S2a, b), while only a minor loss of PD-FcY was observed (Figure S2c). Thereafter, a protein precipitation step with ammonium sulfate was carried out in order to concentrate proteins and change the buffer. Also this procedure did not result in appreciable PD-FcY losses (Figure S2d).

To purify PD-FcY from the clarified extract, two affinity-purification methods (Ligatrap IgY and Genscript IgY) were then tested. Fractions eluted from the Ligatrap IgY column containing the highest concentration of PD-FcY (fractions 2 to 7; Figure S3a) were pooled together. Western blot analysis of the pool separated under non-reducing conditions (El) revealed the presence of two bands migrating at about 100 kDa and two bands migrating at about 50 kDa (Fig. 2a, left panel). Identical experiments performed under reducing conditions showed only two major bands migrating at about 48 and 45 kDa, corresponding to intact and degraded forms of PD-FcY, respectively (Fig. 2a, right panel). The difference in the intensity of the signal observed in the extract (Ext) and in the flow through (FT) demonstrated the binding efficiency of the resin. ELISA experiments performed with an antibody specific for the N-terminal portion of the chimeric protein confirmed the presence of the intact PD in the purified samples (Figure S3b).

The Ligatrap IgY purified sample was dialysed, concentrated (without significant recombinant protein loss) (Dia; Fig. 2a), and further characterized by SDS-PAGE performed under reducing conditions followed by Coomassie staining (Fig. 2b). This analysis demonstrated that both the PD-FcY sample and p19 negative control (proteins eluted from the column loaded with the p19-plant extracts) exhibited a complex pattern of bands. However, two equally abundant bands migrating at about 48 kDa (intact PD-FcY monomer) and 45 kDa (putative degradation fragment) were evident only in the PD-FcY sample (Fig. 2b). From the analysis of the band intensities, the calculated yield of these PD-FcY forms (representing 20% of the total purified proteins) was about 6 mg per kg of fresh leaves weight.

Western blot analysis of the sample eluted from the Genscript IgY resin (El; Fig. 2c) showed a pattern of bands similar to that obtained with the Ligatrap IgY column both under reducing and non-reducing conditions. In addition, no signals were detected in the column FT, indicating a very high binding efficiency (Fig. 2c). Unfortunately, an efficient elution of the recombinant protein was obtained only by boiling the resin beads in SDS-PAGE sample loading buffer, thus making the final product unsuitable for functional characterization purposes. Based on these results, the Ligatrap-purified PD-FcY was used for further functional characterization experiments.

Proteomic and glycosylation analysis

In order to characterize the modification status of the two putative *N*-glycosylation sites occurring in PD-FcY (Fig. 3a), the recombinant product obtained using the Ligatrap IgY column was further purified by immunoprecipitation with the Genscript IgY resin taking advantage of its high selectivity. The eluted material was resolved by SDS-PAGE under reducing conditions and four main bands were detected (Fig. 3b), two corresponding to the heavy and light chain of the capture goat antibody released from the resin (also present in the p19 control sample), and two at about 48 kDa (band 1) and 45 kDa (band 2), corresponding to PD-FcY products. Proteomic analysis of band 1 and 2 identified the corresponding migrating components as intact and C-terminal degraded forms of PD-FcY, respectively (Figure S4, Table S1), based on peptide mapping data showing the absence of specific peptides at protein C-terminus in the latter species. Glycosylation of the intact PD-FcY (band 1) was determined by nLC-ESI-Q-Orbitrap MS/MS analysis of the corresponding endoprotease Asp-N digest, followed by database search of the resulting mass spectrometry data with Byonic™ software. Assignment to specific *N*-linked glycan structures was based on mass fragmentation data. This experiment evidenced the presence of two glycosylation sites, namely Asn236 and Asn335, which are present on the Cυ2 and Cυ3 of the Fc part of PD-FcY, respectively (Fig. 3a). Specifically, glycosylation analysis revealed the presence of the peptide SLSSRV*N*VSGT (Asn236) bearing plant-type *N*-linked glycostructures including α-1,3-fucose (F) and β-1,2-xylose (X) residues, as well as of the peptide EHF*N*GTYSASSAVPVSTQ (Asn335), showing an uncommon plant-type glycoform with high-mannose moieties (Table S2). A low percentage of non-glycosylated forms were found for both peptides, and interestingly, only Asn335 was found to be deamidated. This peculiar feature could be explained by results of previous studies showing that in physiological conditions (neutral pH), Asn followed by a Gly has a strong tendency to be deamidated (Kameoka et al. 2003). The estimation of relative abundance of the different glycoforms was obtained by measuring the area of the extracted ions (XICs) of the identified glycopeptide species from nLC-ESI-MS chromatograms. The relative abundance of the different glycoforms for the two glycosylation sites is reported

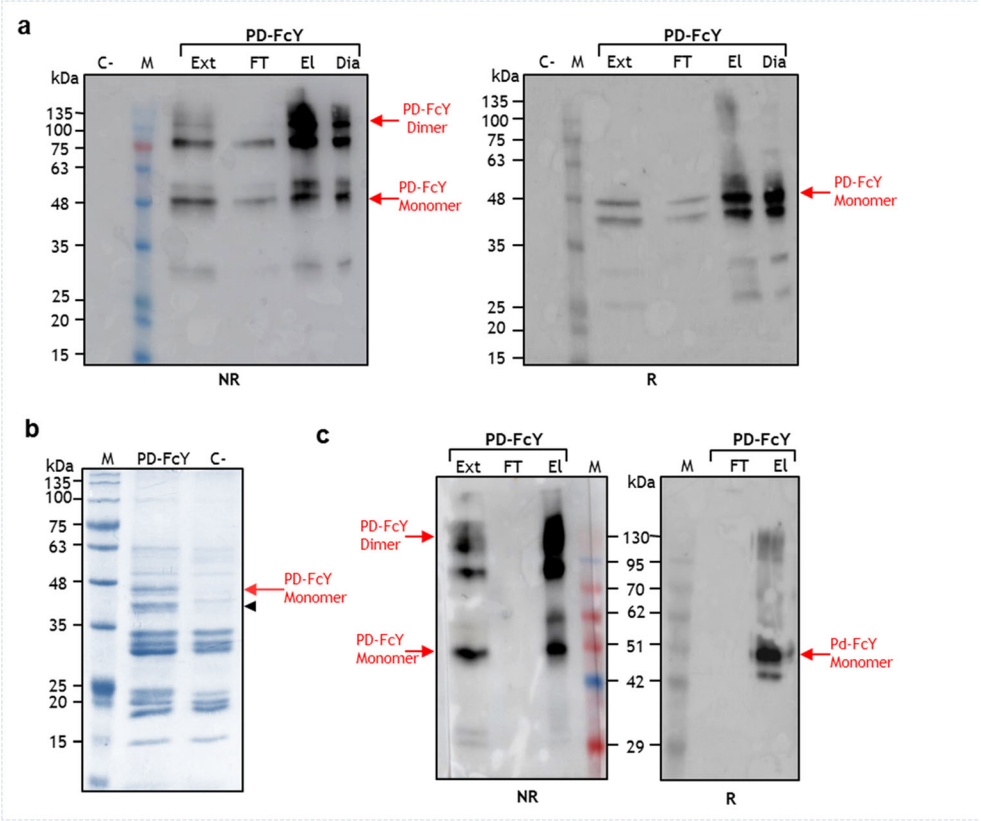


Fig. 2 Purification of PD-FcY using affinity chromatography methods. **a** Western Blot analysis with an anti-IgY antibody of a pool of the fractions (2 to 7) eluted from the Ligatrap IgY column (El). Ten microliters was separated by 12% SDS-PAGE under non-reducing (NR, left panel) and reducing (R, right panel) conditions. C−: pool of the fractions (2 to 7) eluted from the Ligatrap IgY column loaded with p19 plant extract (20 μl); M: molecular mass marker; Ext: clarified extract (20 μl); FT: column flow through (20 μl); Dia: PBS dialysed PD-FcY eluted fractions (concentrated two times) (5 μl). **b** Coomassie staining of proteins eluted from the Ligatrap IgY column and separated by 12% SDS-PAGE under reducing conditions. Twenty microliters (7 μg of total purified proteins) was loaded in each well. M: molecular weight marker; C−: proteins eluted from Ligatrap IgY column loaded with p19 clarified plant extracts. Red arrow: PD-FcY monomer. Black arrow head: putative PD-FcY degradation product. **c** Western blot analysis with an anti-IgY antibody of the proteins eluted from the Genscript IgY resin. Samples were separated by 10% SDS-PAGE under non-reducing (NR, left panel) and reducing (R, right panel) conditions. M: molecular mass marker; Ext: clarified extract (20 μl); FT: resin flow through (20 μl); El: resin elution (5 μl)

in Fig. 3c. The analysis of the PD-FcY degradation product (band 2) showed a glycosylation pattern of Asn335 very similar to that of the intact form, while minor differences in the relative abundance of some glycoforms were determined for Asn236 (data not shown).

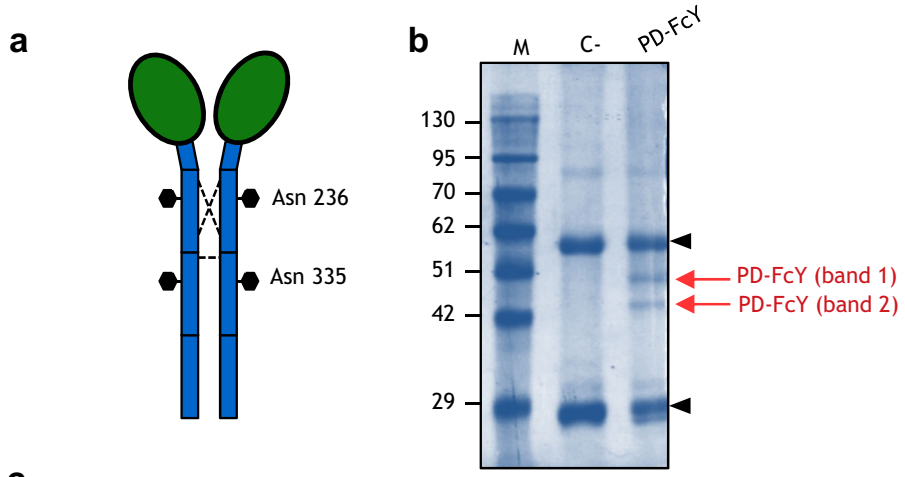
PD-FcY binding to the CHIR-AB1 avian receptor

To evaluate the binding of PD-FcY to the Fc receptor, an assay based on β-galactosidase activity in mammalian BWZ.36-reporter cells expressing the chicken Fc receptor CHIR-AB1 (BWZ.36-CHIR-AB1) was performed. When the cells were stimulated with the purified PD-FcY, high levels of β-galactosidase activity were observed (Fig. 4). The response was similar in strength to that obtained stimulating the cells with a native IgY, with the CHIR-AB1-specific monoclonal antibody (mAb) 8D12, or with the phorbol myristate acetate (PMA), used as controls. As expected, no significant stimulation was observed in the cells treated with the p19 control, or left untreated.

Immunogenicity and viral challenge studies

To verify the antigenic potential of the plant-produced chimeric protein, an ELISA assay was set up in which the ability of a rabbit anti-IBDV serum to recognize PD-FcY was tested. Results from this experiment clearly demonstrated that the PD domain is bound by antibodies raised against the whole virus (Fig. 5a).

Antigenicity of partially purified plant-produced PD-FcY was further tested by intra-muscular administration to specific-pathogen-free (SPF) chickens. Chickens were immunized at day 10, 17, 24, and 31 after hatching with 8 μg PD-FcY (40 μg of total purified proteins) plus adjuvant and challenged with vvIBDV at day 41 (PD-FcY group) (Fig. 5b). Two groups of chickens were injected following the same immunization schedule with the commercial inactivated IBDV



c

Peptide (230-240) SLSSRV*N*VSGT				
Glycan composition	**Glycoform**	**Glycan modification mass (Da)**	**Observed mass (MH^+) (Da)**	**Relative abundance (%)**
Non-glycosylated	-	-	1106.58	3.6
GlcNAc(1)	GlcNAc only	203.08	1309.66	21.4
GlcNAc(2)Man(3)Fuc(1)	Paucimannose: coreF	1038.37	2144.95	1.6
GlcNAc(2)Man(3)Fuc(1)Pent(1)	Paucimannose: coreXF	1170.42	2276.99	27.8
GlcNAc(3)Man(3)Pent(1)	Complex: GnX	1227.44	2334.02	2.5
GlcNAc(3)Man(3)Fuc(1)	Complex: GnF	1241.45	2348.03	1.5
GlcNAc(3)Man(3)Fuc(1)Pent(1)	Complex: GnXF	1373.50	2480.08	24.1
GlcNAc(4)Man(3)Fuc(1)Pent(1)	Complex: GnGnXF	1576.58	2683.15	17.4

Peptide (332-349) EHF*N*GTYSASSAVPVSTQ				
Glycan composition	**Glycoform**	**Glycan modification mass (Da)**	**Observed mass (MH^+) (Da)**	**Relative abundance (%)**
Non-glycosylated, N335-deamidated	-	-	1882.85	8.8
GlcNAc(1)	GlcNAc only	203.08	2084.94	7.0
GlcNAc(2)Man(7)	High Mannose M7	1540.53	3422.39	3.0
GlcNAc(2)Man(8)	High Mannose: M8	1702.58	3584.43	11.1
GlcNAc(2)Man(9)	High Mannose: M9	1864.63	3746.50	32.2
GlcNAc(2)Man(9)Glc(1)	High Mannose precursor : M9Glc	2026.69	3908.55	37.5
Others		*		0.4

Fig. 3 Proteomic characterization and glycosylation analysis of the PD-FcY fusion protein. **a** Schematic representation of the PD-FcY dimer highlighting the two putative (and ascertained) glycosylation sites at Asn236 and Asn335 (shown as black hexagons). **b** 10% SDS-PAGE analysis under reducing conditions followed by Coomassie staining of the PD-FcY or the p19 (C−) eluted samples (20 μl). M: molecular mass marker; PD-FcY: PD-FcY eluted sample. Black triangles: bands corresponding to heavy and light chain of the goat antibody released from the resin. **c** Glycopeptides identified in the endoprotease Asp-N digest of purified intact PD-FcY following analysis with nLC-ESI-Q-Orbitrap MS/MS and Byonic search of resulting data. Assignment of glycan structures was based on measured glycopeptide mass values, fragmentation spectra, and *N*-linked oligosaccharide structures conventionally detected in eukaryotic organisms. Mean values from two independent biological replicates are shown. Glycopeptide species presenting a percentage abundance value below 1% were not reported singularly, but were represented in others: *N335*[+2189]; *N335*[+2205]

vaccine NOBILIS® GUMBORO inac (positive control-vaccine group) or with 40 μg of total purified proteins of the p19 negative control (p19 group). Serum samples were collected from chickens 1 day before each immunization (day 9, 16, 23, and 30) and before the viral challenge (day 41). Individual responses were evaluated using the commercial ProFLOK® IBD PLUS ELISA kit, which measures antibodies specific to IBDV VP2 protein in the serum. The results indicated that the rise of antibody levels induced by PD-FcY was delayed as compared with that induced by the vaccine; in fact, only one positive animal out of 5 was observed for PD-FcY at day 23, while 3 out of 5 were observed for the vaccine at the same time point. Moreover, the antibody levels present in the positive samples at each time point were

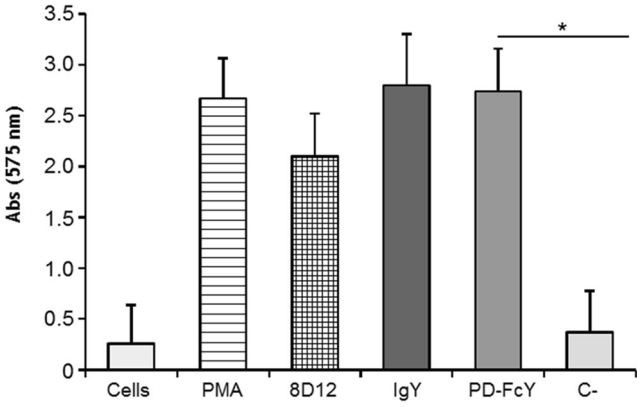


Fig. 4 Binding of PD-FcY to the CHIR-AB1 avian receptor. Measurement of β-galactosidase activity in BWZ.36-reporter cells expressing on their surface the chicken Fc receptor CHIR-AB1 stimulated with PD-FcY (PD-FcY) or the purified p19 extract (C−) used as a negative control. As positive controls, cells were stimulated with PMA, CHIR-AB1-specific mab 8D12 (8D12), and native chicken IgY (IgY). Unstimulated cells (cells) were used as internal assay control. Mean ± SD of three independent assays is shown. *Student's *t* test, $p < 0.05$ for PD-FcY sample versus C−

always significantly higher in the sera of animals belonging to the vaccine group. Nevertheless, 4 out of 5 PD-FcY-injected animals produced significant levels of VP2-specific antibodies at day 41 (Fig. 5c). As expected, all chickens of the vaccine group were still alive 10 days after the viral challenge. Interestingly, this was also true for the PD-FcY immunized chicken no. 3 which showed the highest anti-VP2 antibody response within the PD-FcY group. The other animals in the group died between day 3 and day 4 after challenge. No VP2-specific antibodies were detected in the p19 control group and all challenged chickens were no longer alive 3 days after the viral infection.

Discussion

Traditional IBDV protection strategies are mainly based on the use of inactivated or live-attenuated vaccines. In particular, the latter pose several safety concerns due to the possibility of selecting novel strains with increased virulence (He et al. 2009). For these reasons, recombinant subunit vaccines are regarded as the most innovative approach not only in terms of safety but also for the possibility of easily discriminating vaccinated from infected animals (Hasan et al. 2016). However, recombinant subunit vaccines still pose several drawbacks compared with traditional ones, mainly represented by their partial immunogenic and protective efficacy, and by the labor intensive production processes that limit their cost competitiveness (Müller et al. 2012).

In the ambitious attempt to develop a cost-effective/needle-free vaccination strategy allowing the mucosal delivery of the antigen inducing both local and systemic immunity, we designed and expressed in plants a chimeric protein based on the fusion of the antigenic projection domain (PD) of VP2 to the Fc of avian IgY. PD was selected because deletion mapping and selection of IBDV escape mutants with neutralizing monoclonal antibodies have indicated that the two outmost and hydrophilic regions of this domain, namely the P_{BC} loop (portion 210–225) and the P_{HI} loop (portion 312–325), are critical for virus neutralization (Letzel et al. 2007). The Fc domain was selected because previous studies in mice demonstrated that chimeric proteins composed of antigens fused to the Fc portion of IgG, which may be considered as a functional homolog of IgY (Taylor et al. 2009), were able to bind to Fc receptors (FcR) in the airway mucosa and, after being transported across the epithelial surface, to induce enhanced immune responses and protection (Yoshida et al. 2004; Kuo et al. 2010). Poor information is available about the distribution and role of FcR for IgY (FcRY) in chickens. Nonetheless, from a functional point of view, a full-length FcRY expressed in polarized mammalian epithelial cells was functional in endocytosis, bidirectional transcytosis, and recycling of chicken Fc/IgY (Tesar et al. 2008). Moreover, recent publications demonstrated that the fusion of antigens to the IgY constant region stimulates the activation of avian immune cells by increasing recombinant antigen uptake and processing by chicken macrophages that ultimately lead to an enhanced immune response (Dong et al. 2016; Wang et al. 2017). Beside immunological aspects, the Fc domain may "stabilize" and increase the expression levels of the recombinant antigen and be used to simplify the purification of the fusion protein circumventing the need to add unwanted protein tags that could alter the immunogenicity and safety of the final product (Czajkowsky et al. 2012).

In this study, a secretory version of PD-FcY was transiently expressed in *N. benthamiana* showing good accumulation levels in leaves of the intact monomeric PD-FcY form as well as of assembled dimers (100 kDa) or high molecular mass multimers. The dimeric and high molecular mass forms were particularly abundant in the plant intercellular fluids, indicating the correct processing and active secretion in the apoplast by the cellular mechanisms of protein synthesis. The occurrence of the intact PD in the plant-expressed fusion protein was validated with an antibody specific for the recombinant protein N-terminus while the recognition by a polyclonal serum against the whole IBD virus demonstrated its immunogenic potential.

To improve PD-FcY recovery from plant extracts and minimize unwanted contaminants, the extraction conditions were optimized and the possibility of exploiting IgY Fc for recombinant protein purification purposes was tested. Unfortunately, traditional affinity chromatography columns based on protein A or G resins could not be used for avian immunoglobulin purification and the thiophilic adsorption chromatography, commonly used to purify IgY from the egg yolk (Belew et al. 1987), did not allow to recover significant amounts of

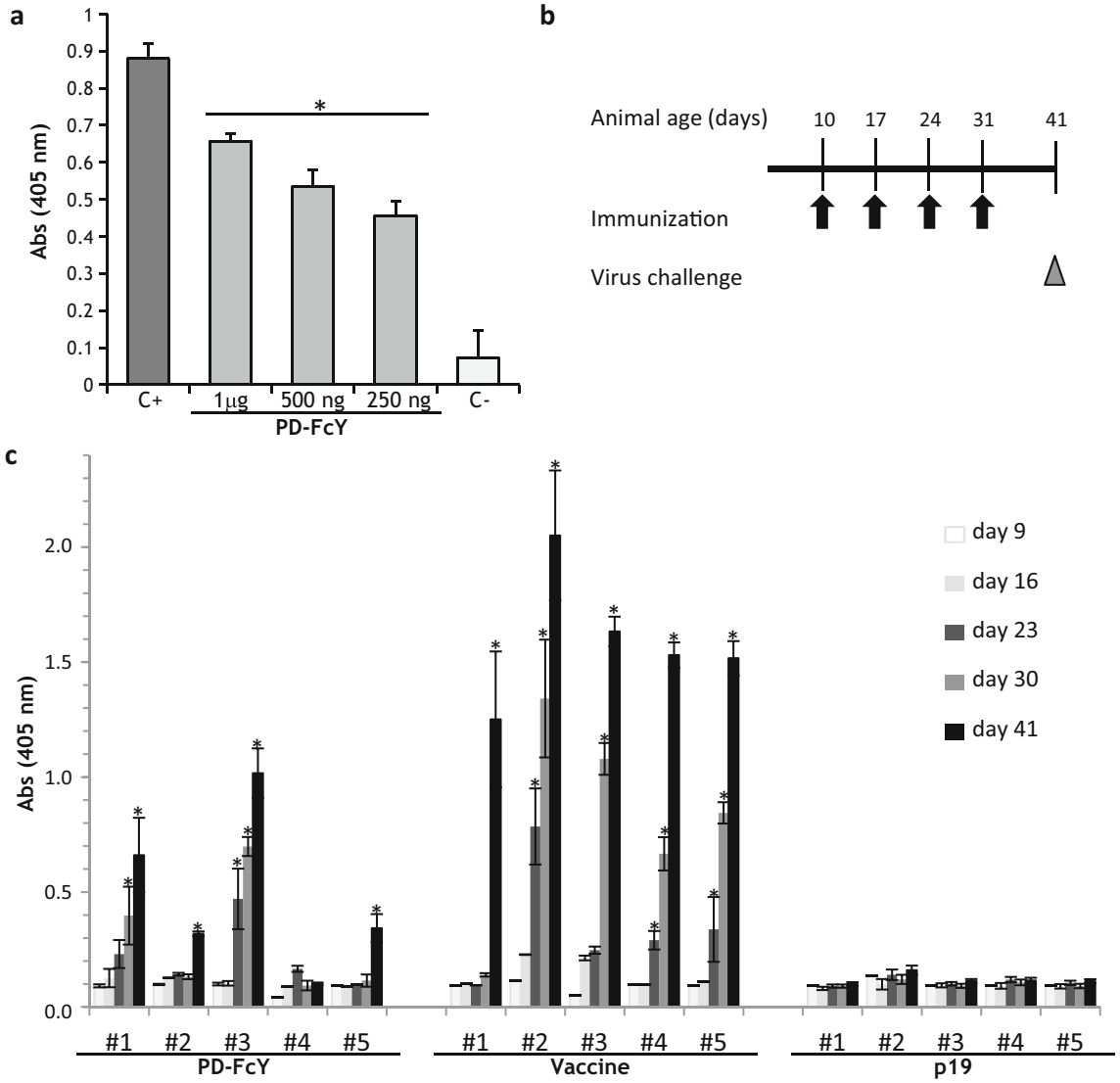


Fig. 5 PD-FcY recognition by anti-IBDV rabbit serum and antibody response in immunized chickens. **a** Direct ELISA of the purified PD-FcY using a rabbit serum anti-IBDV. PD-FcY: dialysed fractions eluted from the Ligatrap IgY (PD-FcY quantity: 1 μg, 500 ng and 250 ng); C−: fractions eluted from the Ligatrap IgY column loaded with the p19 plant extracts (12.5 μl); IBDV: inactivated IBDV (1 μl). Values are the mean ± SD of triplicate wells. *Student's *t* test, $p < 0.05$ for all PD-FcY samples versus C−. **b** Schematic representation of the chicken immunization schedule. **c** ELISA of the sera of immunized chickens. Animals were immunized intramuscularly with PD-FcY, an IBDV commercial vaccine (vaccine) (positive control) or p19 (negative control) at day 10, 17, 24, and 31 of age and sera were collected 1 day before immunization and just before the viral challenge at day 41. The presence of IBDV-specific antibodies was determined using a commercial ELISA kit. Results show mean absorbance values (OD_{405}) ± SD of triplicate wells for each animal. *$p < 0.05$ compared with the p19 control samples (two-tailed, unpaired Student's *t* test and one-way ANOVA)

the plant-expressed PD-FcY (Figure S5). Thus, alternative IgY-specific methods based on immunoprecipitation or affinity column chromatography were assayed. The most selective purification method tested was based on immunoprecipitation of the fusion protein using a resin coupled with antibodies recognizing the Fc region of IgY (Genescript IgY). However, in this case, efficient PD-FcY release from the resin was only obtained using harsh conditions (e.g., boiling in SDS-PAGE sample buffer), and the final recovered product could not be used for immunization purposes. The purification strategy adopted in this study is based on the Ligatrap IgY resin, which is allowed to obtain acceptable recovery yields of partially purified PD-FcY (in the range of 6 mg per kg of fresh leaves). In this case, the characterization of the fusion antigen under reducing conditions evidenced the occurrence in the preparation of two equally abundant molecular species, one corresponding to the intact PD-FcY (band at about 48 kDa) and a smaller one (band at about 45 kDa) corresponding to a degradation product lacking a C-terminal portion of the Fc Cυ4 domain. The degradation of the constant region of mammalian antibodies by plant proteases is a well-known process (Hehle et al. 2015), and this is the first evidence of the

proteolytic processing of an avian IgY expressed in plants. Purified PD-FcY was further characterized for both glycosylation and functionality. Mass spectrometry analysis showed that the Fc region was N-glycosylated at the expected sites (residues Asn236 and Asn335), the first (present in Cυ2) showing typical plant complex sugars, while the second (in Cυ3) showing high-mannose-type oligo-saccharides. This peculiar glycosylation pattern, which is very similar to the one normally observed in IgY from chicken serum, is thought to play an important role in the correct protein folding and stability (Sheng et al. 2017; Suzuki and Lee 2004). In fact, it has been hypothesized that in IgY, after the addition of high-mannose-type sugars to Asn407 (corresponding to Asn335 of PD-FcY) in the endoplasmic reticulum, the Cυ3 domain is rapidly folded and this glyco-site becomes inaccessible to other glycan-processing enzymes in the Golgi apparatus (Suzuki and Lee 2004). Therefore, the presence of high-mannose sugars found on Asn335 of PD-FcY indirectly validates the correct folding of the Fc region of the fusion protein produced in plants. Interestingly, a similar high-mannose glyco-site was also observed in the Cε3 domain of a plant-produced IgE (Montero-Morales et al. 2017) confirming that IgE and IgY share similar structures (Taylor et al. 2009).

The correct glycosylation, folding, and assembly of the Fc region would be fundamental to allow the uptake of the chimeric antigen by specific receptors such as CHIR-AB1 and FcRY (He and Bjorkman 2011; Purzel et al. 2009). CHIR-AB1 is a receptor expressed on chicken B cells, macrophages, monocytes, and NK cells, and its binding to the IgY Fc activates the immune response in chicken (Viertlboeck and Göbel 2011). In this work, we demonstrated the functionality of the Fc by testing the binding of the purified PD-FcY to the CHIR-AB1 receptors in vitro, using a mammalian reporter cell line expressing this avian receptor. The results of these experiments clearly showed that the fusion protein binds to CHIR-AB1 at levels comparable with IgY from chicken serum or with a receptor-specific mAb, indicating that PD-FcY has full potential to stimulate avian immune cells.

Nonetheless, because this was the first time that the PD domain of VP2 was expressed for vaccination purposes, its ability to induce the production of antibodies able to recognize IBDV had also to be confirmed. To this aim, a preliminary experiment was set up in which chickens were intramuscularly injected with the fusion protein supplemented with an adjuvant. The delivery route and the immunization schedule adopted at this initial stage were aimed solely to demonstrate the antigenic potential of PD, herein used for the first time as an immunogen. The results indicated that PD-FcY induced the production of antibodies able to recognize VP2, but also that the elicited response was less efficient and delayed in time as compared with that elicited by the vaccine. It must be noted that the vaccine is made of inactivated virus particles whose coat is made exclusively by the VP2 protein (Coulibaly et al. 2005), the only IBDV antigen able to induce protective immune responses (Letzel et al. 2007; Pitcovski et al. 1999), of which PD represents a small portion. In a previous study using the whole VP2 antigen self-assembled into subviral particles (SVP), good levels of neutralizing antibodies were obtained by delivering 4 doses of 20 μg of SVP (Taghavian et al. 2013). Therefore, a possible explanation for the different efficacy of PD-FcY compared with the inactivated vaccine could be that the injected dose (8 μg) was suboptimal. This hypothesis is strengthened by the observation that among the four PD-FcY immunized chicks producing VP2-specific antibodies, the chicken showing the highest antibody levels (similar to those observed in the vaccine-injected animals) was the only one to survive to the viral challenge. Considering that PD, the inactivated viral vaccine, and the viral strain used for the challenge share the same amino acid sequence in the two major VP2 antigenic determinants (PBC and PHI loops), the obtained results indicate that PD-FcY was able to induce the production of antibodies endowed with neutralizing properties, confirming the correlation existing between anti-VP2 antibody levels and animal protection (Jackwood et al. 1999). In order to verify the applicability of PD-FcY as a vaccine, future experiments will be aimed at determining the dose required to induce protection according to a conventional vaccine administration schedule, the immunogenic efficacy following mucosal administration, and the corresponding efficacy in the absence of adjuvants.

In conclusion, overall results demonstrated that the PD-FcY fusion antigen can be successfully produced in plants and may be regarded as a promising component for the formulation of a low-cost IBDV subunit vaccine, potentially allowing to differentiate infected from vaccinated animals. Further experiments will be necessary in order to characterize in detail the immunological properties of this antigen construction strategy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal experiment procedures were conducted in strict accordance with the Decree of the Italian Ministry of Health n. 26 of 4 March, 2014, on the protection of animals used for scientific purposes, implementing Directive 2010/63/EU, and approved by IZSVe's Ethics Committee or in accordance with European and French legislations on laboratory animal care and use (French Decree 2001-464 and European Directive CEE86/609) and animal protocols approved by the Ethics Committee "Sciences et santé animale," committee number 115. The animals were kept within the animal facilities (biosafety level 2) of IZSVe or of Agronomy and Veterinary Institute Hassan II.

This article does not contain any studies with human participants performed by any of the authors.

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